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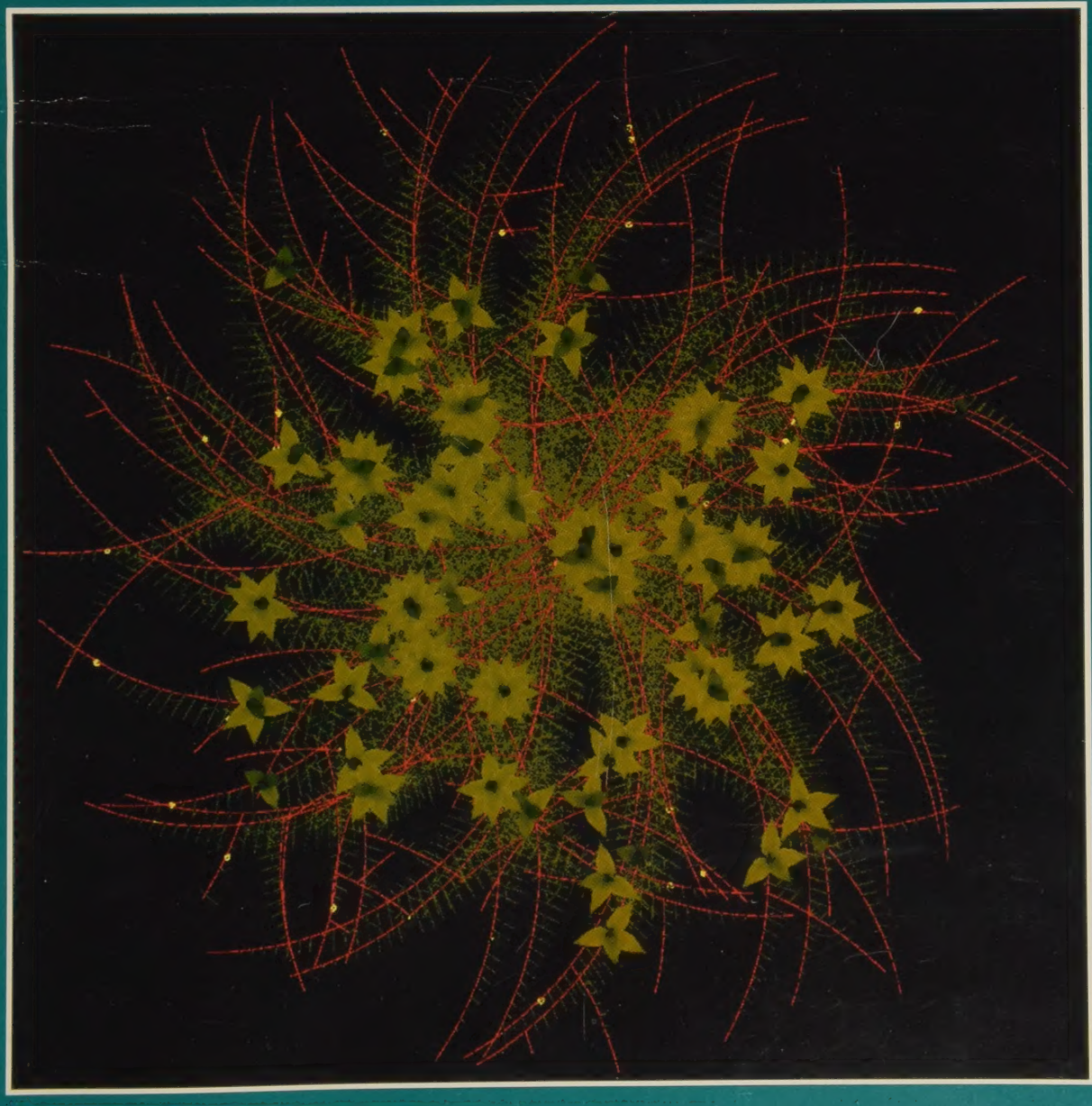
Modern botanical research using plants of ancient lineage



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GRAY HERBARIUM
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John Torrey
1796 – 1873

The Torrey Botanical Club came into being after a group interested in botany began, in 1867, to meet regularly at the Herbarium at Columbia College where John Torrey, M.D. was a Professor of Botany and Chemistry. This painting of John Torrey, by the noted American portrait painter Henry Inman, was completed in 1833 during the time of Torrey's professorship at the University of the City of New York, now NYU.

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The Torrey Botanical Club

EDITORS' INTRODUCTION

This issue is comprised of papers presented in a Symposium celebrating the 125th anniversary of the Torrey Botanical Club held in August 1992 and dedicated to the memory of one of its more distinguished members and past presidents, William J. Robbins. It may seem incongruous that all the papers in this Symposium report on research conducted with bryophytes. The Torrey Botanical Club is not a bryological society. William Robbins was not a bryologist. Nevertheless, we see relationships. The Torrey Botanical Club is the oldest botanical society in the Western Hemisphere that has been continuously active in gathering and distributing information about plants. It evolved from the locally active "Metropolitan Botanical Club" to an internationally prominent scientific organization. The Bryophyta is possibly the oldest phylum of extant photosynthetic land plants that has evolved to become diverse in composition and cosmopolitan in distribution. The "Torrey Club" was founded for and is committed to the pursuance and enhancement of all areas of botanical research. The bryophytes have proven to be challenging and rewarding subjects for almost all of these areas. William Robbins was primarily a physiologist. The objects (i.e. fungi to flowering plants) and subjects (i.e. antibiotics to anthocyanin formation) of his research were diverse and the results far-reaching. Most of his many papers would, nevertheless, be appropriate for publication in the Bulletin of the Torrey Botanical Club. Apropos of this Symposium, he published one of the earliest papers concerning the use of bryophytes in axenic culture for studying plant nutrition. This Symposium, above all, celebrates the individuals like William J. Robbins, who make for enduring societies and plants such as the bryophytes that help reveal the unities underlying the diversity of plants and plant processes.

The Editors

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Modern botanical research using plants of ancient lineage

Prepared for a symposium convened to celebrate the 125th anniversary of the Torrey Botanical Club.

Edited by Dominick V. Basile and Paul S. Mankiewicz

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Modern research with bryophytes: an overview [1]

Norton G. Miller
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MILLER, N. G. (Biological Survey, New York State Museum, Albany, NY, 12230). Modern research with bryophytes: an overview. Mem. Torrey Bot. Club 25(1): 1–10. 1993. – The idea that bryophytes form a truly ancient lineage of plants rests upon inadequate fossil evidence of their presumed lower Paleozoic origins from Charalean-like or Coleochaetalean-like algae. In this regard a special challenge is the unequivocal interpretation of the biphasic life cycle of bryophyte-like plant fossils. Unique among land plants in having a dominant gametophyte phase, bryophytes present opportunities for a broad array of research not readily undertaken in sporophyte-dominated organisms. This is enhanced by the ease with which bryophytes in culture can be manipulated experimentally and subjected to analytical testing. Such attributes have permitted numerous recent advances in what is known about the cell biology and physiology of this group of plants. Progress is also evident in deciphering the natural products chemistry of bryophytes. Of far reaching importance are recent advancements in understanding speciation and genetic diversity within species. Where bryophytes are vegetation dominants (e.g., polar, arctic, and boreal regions, and low latitude-high elevation forests), the importance of research involving these organisms will increase in proportion to environmental pressures created by the expanding human population and its agricultural and industrial base.

Key words: bryophyte epiphytes, fossil phytodebris, land plant fossils, molecular systematics, moss banks, *Sphagnum leucobryoides*, mycorrhizae, *Takakia*.

There exist two general approaches to research involving bryophytes. The first focuses on the innate characteristics of these organisms, where they occur, what are their evolutionary relationships, what aspects of their biology are special or unique, and so on. The second involves the use of bryophytes as model systems in an effort to understand general biological principles.

The scope and intensity of research of both kinds can be appreciated by inspection of the published results of this work. *Journal of Bryology*, which is sponsored by The British Bryological Society, and *The Bryologist*, a publication of the American Bryological and Lichenological Society, contain comprehensive lists of the recent bryological literature. About the same number of bibliographic entries are provided in each issue, but because *The Bryologist* is published quarterly, and the *Journal of Bryology* is biannual, twice as many entries (about 1400) are given in *The Bryologist* per year than in the *Journal*. In *The Bryologist* the bibliographies are presented as separate lists for mosses and for hepatics and hornworts. In the *Journal* the list is divided into subjects and includes the following headings: general (ecology, phytogeography, taxonomy); anatomy, morphogenesis, physiology; chromosome cytology; biochemistry; and pollution. In the bibliographies published between 1988 and 1992 roughly 70% of the listed papers are taxonomic, floristic, or ecological; 19% deal with the anatomy, development, and physiology of bryophytes; 6% with biochemistry (with

[1] Prepared for a symposium, "Modern Botanical Research Using Plants of Ancient Lineage," convened to celebrate the 125th anniversary of the Torrey Botanical Club. I and other botanists of the New York State Museum's Biological Survey feel a special kinship to the eponymous John Torrey who was appointed the first botanist of the Survey shortly after it was founded in 1836 by the State Legislature. His charge was to explore New York State and to document its plant life. Torrey's labors resulted in the first flora of New York, published in 1843, a two-volume, illustrated work of large format, treating some 1450 species of seed plants. Voucher specimens documenting this project, originally mounted in 50 folios, initiated what has become a large and important herbarium kept by the New York State Museum and at which botanical research continues.

I thank David K. Smith for information about *Takakia ceratophylla*.

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the identification of secondary metabolites the major component); 2% with chromosomes or nuclear genomes; and 4% with a well characterized branch of applied bryology, the use of bryophytes as indicators or evidence of pollution. I have been unable to discern any trends in the numbers of entries in these categories since 1988, which indicates to me that there has been continuous progress on (and a consistent interest in) all of these aspects of bryological research.

The systematics, floristics, and ecology of bryophytes have not been featured in the symposium of which this paper is a part, but such studies clearly occupy a substantial proportion of the research endeavors of contemporary bryologists. There are many reasons why this is so, but one of the most important is that the bryoflora of the world is incompletely known. It is generally accepted that there exist about 14,000 kinds of mosses, 8000 liverworts, and 400 hornworts, but these estimates may be reduced as taxonomic revisions are completed. For example, Dries Touw (1974) has reckoned that there may be only 7000 mosses on the basis of an extrapolation of trends in the number of species accepted in published monographic studies, particularly those that include reappraisals of all proposed species in a genus and analyses of morphological variation in plants collected over a broad area. Paradoxically perhaps, new and clearly marked species continue to be discovered, described, and accepted, in fact many each year, as are previously unknown life-cycle components of recognized species. Even in the European flora, probably the best-known part of the world bryologically, additions and new species are found regularly (e.g., Boudior & Pierrot, 1992; Hodgetts & Blockeel, 1992). Thus, there is an urgency to continue exploration vigorously everywhere before habitat degradation and destruction result in important losses from the world's bryoflora.

Two examples of important recent bryological discoveries make this point clearly. The first is finding the long-elusive antheridial plants of *Takakia ceratophylla*. As luck would have it, they were growing associated with archegonial plants, a mingling that allowed the production of the also previously unknown sporophytes. The discovery was made on Atka Island, Aleutian Islands, Alaska, by David Smith and Paul Davison of the University of Tennessee (Davison *et al.*, 1989; Smith, 1990).

The classification of *Takakia* (a genus of two species) has been unsettled. Many workers have considered it a hepatic allied to *Haplomitrium* and *Calobryum,* with which it shares radial, upright gametophore symmetry and a horizontal system of subterranean leafless stems. Previously unknown elements in the life cyle of *T. ceratophylla*, including a calyptra that is carried aloft as the sporophyte lengthens, antheridial development and dehiscence, spermatid ultrastructure, and other anatomical details elucidated by David Smith, Karen Renzaglia, and their collaborators (McFarland *et al.*, 1989; Smith *et al.*, 1990; Renzaglia *et al.*, 1991), establish that *T. ceratophylla* is a moss, probably best placed close to *Andreaeobryum*, an assignment advocated by Barbara Murray (1988) in a study of the systematic anatomy of the equally bizarre *Andreaeobryum macrosporum* and of *Andreaea.* A more complete discussion of the morphology of *T. ceratophylla* is presented by Smith and Davison (1993). When we are reminded that *Takakia* has been postulated, and with good reason, to be a primitive bryophyte, these new discoveries will prove important in reconstructions of phylogeny.

A second example is *Sphagnum leucobryoides* from Tasmania, described as the sole member of *Sphagnum* section *Buchanania* by Yamaguchi, Seppelt, and Iwatsuki (1990). Unusual features of this 2-cm-tall peat moss include "short" and "long" erect branches (there are no pendent ones!), dimorphic chlorophyllose cells in the short branches, archegonia terminal on the stem, perigonia lateral on the stem just below the perichaetium (vs. on separate branches of the same or different plants in other

Sphagna), and oblong-ellipsoidal antheridia (vs. globose ones in all other *Sphagna*). These character states are unique in *Sphagnum* (which is largely a Northern Hemisphere genus), and together they and perhaps yet to be discovered additional features of this plant may provide sufficient reasons for recognizing this peat moss as a second genus in the Sphagnaceae. The habitat of the species, "buried in sand," is equally unusual.

These examples, just two of many worth singling out, indicate the potential significance of what must still exist undetected in poorly known bryofloras. Undoubtedly many important discoveries will be made during floristic exploration in the future, assuming that such work can keep ahead of pressures that result in the loss of habitats as a consequence of human population growth and expansion into unexploited parts of the earth.

I continue my review of bryophytes in modern botanical research by exploring the meaning of the phrase "plants of ancient lineage," which is part of the title of this symposium. The fossil record provides evidence that the metzgerialean Hepaticae were represented by plants similar in appearance to the extant *Pallavicinia* in the Upper Devonian of New York State (Hueber, 1961). This is the oldest, undisputed fossil bryophyte, but we know little about it other than its general cellular makeup. We assume by analogy to the modern flora that it is a gametophyte, but there is no direct evidence of this. The oldest known fossil moss is from the Lower Carboniferous of England (Thomas, 1972), and fossils assignable to the hornworts are first encountered as probable spores from the Cretaceous of North America (Jarzen, 1979). When the ages of these fossils are compared with the known record of well-documented megascopic fossils of presumed vascular plants, which are from Silurian rocks (Edwards *et al.*, 1983), some 60 million years older than those of the Upper Devonian, or spore tetrads and single trilete spores presumably representing land plants (Gray *et al.*, 1982) in Late Ordovician deposits, 20 to 30 million years older still, there is a huge gap in the fossil record, for which information appears to be lacking on bryophyte origins. It is worth stressing that cladistic phylogenetic reconstruction generally shows the major groups of bryophytes (mosses, liverworts, and hornworts) emerging as separate clades, a concept that should be kept in perspective when considering bryophyte origins and in seeking progenitors for bryophytes in the fossil record.

Despite these cautions there have been some remarkable advances in what is known about the early evolution of land plants. These in part are a result of studies of fossils from the Lower Devonian Rhynie Chert of Scotland in which skillful etching of sectioned permineralized axes and the use of scanning and transmission electron microscopy have revealed new structural details. One of the most intriguing recent discoveries is the existence of a protracheophyte grade of plant organization, exemplified by *Aglaophyton, Nothia*, and other fossils in which the walls of the internal water-conducting cells are like the hydroids of mosses and hepatics (Edwards, 1986; Kenrick & Crane, 1991). Protracheophytes had erect axes, with multiple terminal sporangia arising from a horizontal system of axes that bore rhizoids in small patches on the lower surface. The Rhynie Chert has yielded plants interpreted as the gametophytes of *Aglaophyton* (i.e., *Lyonophyton*) and *Nothia* (i.e., *Kidstonophyton*), which have been reconstructed from serial sections of the fossils (Remy & Hass, 1991a, b). Except for the gametangiophores, they appear to have been isomorphic with the corresponding sporophytes.

These new studies point to the existence of four kinds of elongated water-conducting cells in Early Devonian plants (Kenrick & Crane, 1991): (1) cells without inner wall thickenings; and cells with secondary walls, including (2) an "S-type" (with a microporate inner layer and internally lacunose spiral thickenings), (3) a "G-type" (annular or helical thickenings that are internally homo-

geneous), and (4) cells with scalariform pitting. No known extant vascular plant has S-type cells, but G-type cells are known in zosterophylls (an extinct group) and early lycopods. The absence of inner thickenings in type (1) cells and the microporate layer of S-type cells are similarities shared with the water-conducting cells of certain mosses and hepatics (Edwards, 1986; Kenrick & Crane, 1991).

In a cladistic analysis of embryophyte phylogeny published in 1991 by the paleobotanists Paul Kenrick and Peter Crane, the protracheophytes and tracheophytes are shown as the sister group to three bryophyte clades (mosses, liverworts, and hornworts), on the assumption that the S- and G-type water-conducting cells are homologous. Their hypothesis conforms with the concept that during the Silurian (or earlier) and the Early Devonian a number of different now extinct plant groups were undergoing adaptation to terrestrial environments. Moreover, it is amenable to refinement as more anatomical (and ultrastructural) data become available about fossils of the lower embryophytes.

The Rhynie Chert has yielded new materials of *Aglaophyton major* (first described in 1920 as *Rhynia major*), which is now known to be a nonvascular land plant through the investigations of David Edwards (1986). The excellent preservation of the plants in silica allowed the conducting strand to be studied in detail. It consists of cells similar to hydroids in the center and others like leptoids located in a zone around them, much like those in polytrichaceous moss gametophytes and sporophytes. It is not clear whether the hydroids of *Aglaophyton* have pits like those found in comparable cells of mosses and hepatics, but in well-preserved material it may be possible to ascertain this.

The reconstruction of *Aglaophyton*, based on studies of plants in growth position, shows a decidedly unbryophyte-like sporophyte, with multiple terminal sporangia and no attached gametophyte. The latter was evidently free-living and of similar appearance to the sporophyte. It is not known whether in this plant the zygotes underwent embryogenesis to develop into a sporophyte that was isomorphic with the gametophyte and attached initially to the gametophyte, but such a circumstance would be unparalleled in the life history of any known extant plant. We must admit to having no paleobotanical evidence of the origin of the following salient features that make plants bryophytic: a dominant and variously elaborated gametophyte; and a subordinate, non-appendaged sporophyte, attached continuously throughout its life to the gametophyte and with a single terminal sporangium. We need new clues to how these characteristics arose and when they did.

Thus, while it is not known yet whether bryophytes are truly as ancient as circumstantial evidence would seem to indicate, enough new information has been presented in the last decade to offer hope that this problem may be nearer a solution. The Rhynie Chert or other deposits in which whole plants, tissues, and cell walls are preserved with little alteration may hold some of the answers.

Before leaving the subject of Paleozoic paleobotany, one other topic of recent research deserves emphasis in the context of increasing what is known about bryophyte fossils. The *Pallavicinia*-like fossils mentioned earlier were discovered among plant fragments resulting from the hydrofluoric-acid digestion of shale. Such "phytodebris" is often ignored in favor of megascopic remains of plants and animals or fossil spores and pollen. Gensel, Johnson, and Strother (1991) have reviewed what is known about highly fragmented plant materials from Ordovician, Silurian, and Devonian rocks and the considerable potential of such fossils to provide new data of evolutionary significance. The fragments commonly include small organs or tissue systems, cuticles, conducting cells, and spores. Bits of animals and more or less complete fossils of small terrestrial arthropods have also been revealed by the acid

maceration of fossiliferous sedimentary rocks, particularly those that represent near-shore or other terrestrial deposits.

To identify and interpret phytodebris it is necessary to have a comprehensive familiarity with comparable materials derived from extant plants, in addition to knowledge of the cellular structure of plant and animal megafossils from rocks of the same age (or older) and the same general paleogeographical location. We are far from having adequate references for the rigorous identification of fragmented fossils, but because many bryophytes (both sporophytes and gametophytes) have cuticles (including epicuticular waxes) and distinctive cell patterns, I see the study of fossil phytodebris as potentially yielding important new paleobryological information.

Plant debris, probably including bryophyte fossils, is likely to have gone unrecognized in sediments younger than those containing a record of the origin of the land flora. Indeed, I have discovered that phytodebris rich in moss fossils is common in Pleistocene deposits of small and large fresh-water lakes in northeastern North America. Moreover, fossil mosses have been recovered from Quaternary deposits elsewhere in the Northern Hemisphere and at other places in the world. These fossils, representing plants that grew in microscale habitats, provide a unique source of information about paleoenvironments and past distribution patterns that reflect stages during the formation of areas occupied by species of the contemporary flora. Of particular interest are species in North America 35,000 to 20,000 years ago that were distant from places where they now occur. These species are presently arctic, subarctic, and boreal plants, but during the late Pleistocene, they occurred well to the south in areas now occupied by prairie or deciduous forest. A good example is the moss *Cinclidium latifolium*, known from a full-glacial deposit in central Illinois and late-glacial lake sediment in Minnesota and now restricted to arctic Canada, 2000 to 4000 km to the north (Miller, 1992). Such major and unexpected differences between the distribution of the contemporary and Pleistocene moss floras suggest that species no longer found in the same area may have grown sympatrically in the past, thereby providing a potential for hybridization and speciation through allopolyploidy, a process only recently recognized in mosses and the subject of a paper in this symposium proceedings by Robert Wyatt and his collaborators.

A synthesis of studies of moss fossils from many Pleistocene deposits in eastern and central North America indicates the richness of glacial and early postglacial moss floras in this region and provides evidence of the rapidity with which mosses migrated to and became established on surfaces freed of glacial ice (Miller, 1984). The deposits contain not only fragments of numerous kinds of mosses (the maximum number of species represented so far in a single deposit is 45) but also pieces of moss plants that had begun to proliferate new stems and rhizoids. Propagules of this sort, dispersed by wind or water, probably played an important role in the spread of species, particularly during the time when trees were absent from the landscape and the vegetation was tundra-like in general appearance and floristic composition. North American Pleistocene moss floras consisted mainly of northern (boreal and arctic) species, but with a small representation of mosses that occur in the contemporary flora mostly in climatically temperate parts of North America, such as the Great Lakes—New England region. Species that show an interrupted eastern North American—western North American pattern were also present on glaciated late-Pleistocene surfaces. The studies indicate that much of the present moss flora of northeastern North America was able to survive in the area adjacent to the southern limit of the continental ice sheet and that species were not necessarily restricted to distant refugia in the Southern Appalachians or beyond.

To expand my review of modern bryologi-

cal research, I turn now to a summary, of necessity a cursory one, of symposium proceedings and review volumes published since 1990. I highlight only a few of the many important discoveries or ideas presented in these formats.

The first of a proposed series of international conferences on the physiology, developmental biology, and genetics of bryophytes took place in 1991 (Marienfeld, 1991). Held in Heidelberg, Germany, "Mosses '91" included sessions on phytochrome action, metabolism, the cytoskeleton and cell organization, growth and differentiation, and molecular and population genetics. For the most part the investigations presented were experimental and laboratory-based. Central to a number of the papers was the chloronema/caulonema/gametophore-bud system, which is an ideal model for studying the roles of phytohormones and other chemical signals in plant growth and development. Other presentations at Heidelberg included accounts of path-breaking research, including new evidence that rhizoids and protonemata of mosses are different manifestations of the same developmental stage and the existence of thigmotropic responses in rhizoids (these by Jamie Goode, *et al.*), the *in vitro* production of bisexual diploid plants of *Marchantia polymorpha* through the induced fusion of protoplasts from male and female plants (reported by Regina Viktor and Martin Bopp; the diploid cells eventually grew into thalli that bore antheridiophores and archegoniophores), further news about the isolation of the gene that directs the synthesis of phytochrome (by Thümmler, using *Ceratodon purpureus*), a progress report on the cloning and sequencing of several plastid-encoded genes and one mitochondrial gene from the moss *Physcomitrella patens* (by Wolfgang Abel), and the production of transgenic plants of *P. patens* by the introduction and integration of bacterial plasmids into the moss (by Didier Schaefer and a co-worker, thereby establishing the first haploid system suited to developing a program of research in "reverse genetics").

In 1990 a full account of the chemistry and chemical taxonomy of bryophytes appeared in print (Zinsmeister & Mues, 1990). This book documents an astonishing increase in information about secondary metabolites in bryophytes during the last two decades and highlights some major differences in the classes of chemical compounds found in mosses, hepatics, and hornworts. For example, Markham (1990), while stressing an inadequate sample base, emphasized the apparent absence of flavonoids in hornworts and the presence of these compounds in many of the mosses and hepatics tested so far. Similarly, Geiger (1990) concluded that biflavonoids, while frequent in many mosses, remained undetected in hepatics and anthocerotes. Moreover, it is now clear that liverworts are rich in secondary metabolites, especially monoterpenoids, sequiterpenoids, and diterpenoids. The functions of secondary metabolites in bryophytes (and in other plants) remain poorly known in general, but they may reduce the penetration of damaging UV radiation (some of the compounds appear to be present only in the cuticle) and may serve as antifeedants against insects and molluscs (thereby reducing the depredations of herbivores).

The realization that bryophytes have a complex and potentially significant chemistry has come too late to be a part of the burst of research on the secondary compounds of plants that took place in the 1960's and 1970's. Therefore, the taxonomic usefulness of the chemistry of bryophytes has yet to be adequately explored, even though it is likely to be considerable, particularly in relation to questions of where systematically problematic genera are best classified. Recent examples are provided by Mues (1990) for *Mylia* (chemistry indicates that it belongs to an isolated monotypic subfamily in the Jungermanniaceae); by Asakawa and co-workers (1980) for *Wiesnerella denudata* (the chemistry of which allies it with the Conocephalaceae); and by Whittemore (1991) who concluded

on the basis of their chemistry that the marchantialean families Corsiniaceae and Targioniaceae are derived (as others had postulated using morphological criteria).

Also published in 1990 was a welcome review of the physiology and biochemistry of bryophytes, assembled and edited by the Indian botanists R. N. Chopra and S. C. Bhatla (1990). Many of the topics summarized in this book were featured in the "Mosses '91" symposium as subjects of continuing research. Ashton and co-authors (1990) discussed the promise of haploid bryophyte tissues for genetic analysis and the ease with which mutant phenotypes can be identified. The process of photomorphogenesis in the early stages of moss ontogeny and the role of the phytochrome system is reviewed by the leading student of this subject, Elmar Hartmann (Hartmann & Weber, 1990). The development of moss protonematal systems, particularly of *Funaria hygrometrica*, in relation to phytohormone activity is set out by the originator of much of what is known about this topic, Martin Bopp, who described (1990) the hormonal system in mosses as one in which the "sequential interaction of auxin and cytokinin [exists] as a main component and perhaps cAMP as an antagonistic system. All of these components have been characterized very clearly. The moss protonema may, therefore, play a very important role in further investigations on the primary action of hormones at the molecular level."

I end this summary of recently published reviews of major research programs in bryology by emphasizing another symposium proceedings, "Bryophytes in Terrestrial Ecosystems," published in 1990 (Jury *et al.*, 1990). Among several important topics reviewed in depth were the distribution of bryophyte biomass in contrasting habitats (an average of 30% of the total biomass in the Arctic and 100% in certain habitats in the Antarctic is accounted for by bryophytes; bryophyte mass increases dramatically with elevation in the tropics) (Russell, 1990; Frahm, 1990); interactions between bryophytes and vascular plants, certain eucaryotic algae, fungi, lichens, blue-green algae, bacteria, and molluscs (During & Van Tooren, 1990); the role of bryophytes in mineral-nutrient cycling in plant communities (how nutrients are taken in and released by bryophytes are still largely not understood) (Brown & Bates, 1990); evidence that the potential for establishment from spores is highly variable and may be species specific in mosses (Miles & Longton, 1990); and the use of bryophytes to monitor atmospheric deposition of heavy metals (concentrations measured in gauges and in moss plants are nearly the same) (Tyler, 1990).

I now update some of these subjects and comment on a few additional topics of current research. I also highlight aspects of current work involving problems that can be pursued only through studies of bryophytes.

The first is molecular systematics. The focus of such studies in bryology to date has been on the use of cytoplasmic ribosomal RNA sequences and chloroplast DNA and ribosomal RNA sequences as clues to evolutionary relationships among bryophyte taxa and between bryophytes and other plant groups containing chlorophylls *a* and *b*. There is at present a small amount of literature on this subject, most of it published in the last few years. Therefore, it is impossible to draw any major conclusions. Indeed, some conflicting results are apparent. Applying cladistic methodology to morphological data, Mishler and Churchill put forward in 1984 and 1985 a hypothesis that the embryophytes were monophyletic, and that among the bryophytes (which they regarded as paraphyletic) the mosses alone were the sister group of the vascular plants, the hornworts of the moss- tracheophyte clade, and the liverworts of all other extant land plants. This topology of clades Mishler and co-workers (1992) have since supported independently by sequence data for 16S and 23S ribosomal genes from chloroplasts of 11 bryophytes, representing many major

taxonomic groups. In contrast Debra Waters and her collaborators (1992), using sequences obtained from large and small subunits of nuclear-encoded ribosomal RNA genes from three bryid mosses, three marchantialean hepatics, and two hornworts, found the following cladistic groupings: the mosses and hornworts are sister groups, the mosses and hornworts together are the sister group to the vascular plants, and the liverworts are the sister clade to all other land plants. The seemingly dramatic differences between these hypotheses are likely to be resolved when more gene trees are available for comparison, the number of taxa under study is increased, and improved methods are devised to decipher the phylogenetic message in gene sequences.

A further intriguing example of the potential of molecular data to provide unexpected evolutionary insights is the discovery by Raubeson and Jansen (1992) that the nonlycopsid vascular plants share an inverted 30-kb segment of chloroplast DNA, while in the lycopsids and bryophytes (data from one moss and one hepatic only) the same segment is not inverted. Because the inversion is present in the various pteridophyte groups (excluding the lycopsids), pteridophytes are unlikely to have been the progenitors of the moss and hepatic clades, in contrast to theories that postulated they were derived from pteridophytes by reduction. Whether hornworts also lack the inversion is not known at present.

Mosses and/or liverworts are major components of the vegetation of high-latitude polar land masses, high-elevation tropical cloud forests, and maritime temperate conifer forests. In particular feather-moss/spruce/larch associations are prominent in boreal Canada and Eurasia, and early work established that nutrients were supplied to the moss ground cover mainly from above as throughfall and precipitation. Carleton and Read (1991) have recently shown that nutrients can also be transferred from moss shoots to tree seedlings via mycorrhizal fungi, using radioactive tracers supplied *in vitro* to plants of *Pleurozium schreberi*, and then by establishing through autoradiography that the radioisotopes were transferred to pine seedlings across a mycorrhizal bridge. The fungi were also observed to colonize portions of moss plants several centimeters back from the apex, where senescence, tissue breakdown, and nutrient release occur during decomposition. These results, if they can be verified in natural associations, would establish that carpet-forming mosses are a previously unknown but essential link in the nutrient cycle in a major forest biome.

A similar advance in understanding the role of bryophytes in nutrient capture and release is Darwyn Coxson's recent study (1991) of moss and hepatic epiphytes in the tropical montane rainforest at La Soufrière, Guadeloupe, where the trees are 3-4 m tall. In this research above-the-canopy precipitation and throughflow were collected in field trials (and under laboratory controlled conditions), frozen, and analyzed for solutes by mass spectrophotometry. The results show that nutrient supply fluctuations in the precipitation are minor compared to the magnitude of the pulse release of solutes to throughflow by bryophytes in the canopy during episodic rainfalls. Solute amounts in pulse releases are also greater than background amounts measured in throughfall when the forests are bathed in mist from clouds. Coxson concluded that his estimates of net yearly nutrient release are highly significant and that the capture and release of nutrients by bryophytes is a "major pathway for nutrient redistribution in tropical montane ecosystems" (see also Coxson *et al.*, 1992). Although not quantified, this redistribution would involve the transfer of nutrients to decomposer and leaf epiphyte communities, with a potentially dramatic effect on their physiological activities.

I turn to my last example, which pertains to bryophyte-dominated habitats where decay is reduced by waterlogging and permafrost (thereby limiting oxidation) and where climate exerts a major influence on

plant growth or results in the exclusion of species. In parts of the Antarctic and Arctic, moss "banks" or "beds" accumulate to a thickness of several meters and can be shown by radiocarbon dating to have grown continuously for much of the last 5500 years. These deep deposits of moss peat allow proxy measurements of changing environmental parameters across the last half of the Holocene. They are important supplements to proxy data obtained from sediments, glacier ice, tree rings, and other continuously accreting systems that have been the principal sources of information about past environmental conditions.

Björck and co-workers (1991) analyzed stratigraphic changes in a 5500-year-old moss bank consisting mainly of *Chorisodontium aciphyllum* (Dicranaceae) and *Polytrichum alpestre* on Elephant Island, Antarctic Peninsula. There the peat accumulated as a result of a balance between factors controlling primary productivity (e.g., warm humid summers, high nitrogen influx) and the expansion of permafrost, which is enhanced by long cold winters. Their analyses showed that the moss bank was ombrotrophic and responded to atmospheric inputs much as raised bogs do. The Elephant Island moss bank was found to contain evidence of three peaks in primary productivity (reflecting three highs in nitrogen influx), which was interpreted as three periods of milder and more humid summers, independent evidence for which comes from the study of stratigraphic samples of lake sediment in the same general region. Additional records such as these will produce a fuller understanding of Holocene climates, the dynamics of nutrient flux, and the timing and intensity of volcanic eruptions in the south polar region, all as a result of the proxy records that are stored in deep deposits of moss peat.

There is still much to be learned. The great unknowns, in my opinion, are knowledge of the full scope of the world's bryological diversity, the lack of an integrated understanding of the complex processes of dispersal and establishment in bryophytes, the genetic structure of hornworts and indeed of mosses and hepatics (although there has been some recent progress on this topic for these two plant groups), the establishment of homologies so that the work of comparative evolutionary biology can be carried forward on a sound basis, and a multitude of problems at the cellular and molecular levels and in how bryophytes function as organisms and in ecosystems. This is a substantial agenda.

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Genetic analysis of phytohormone action and morphogenesis in *Physcomitrella patens* [1]

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ASHTON, N. W., T. A. ZACHARIAS AND D. F. FRACCHIA (Departments of Biology and Computer Science, University of Regina, Regina, Saskatchewan S4S 0A2, Canada). Mem. Torrey Bot. Club 25(1): 11–20. 1993. – The analysis of developmental mutants of *Physcomitrella patens* (Hedw.) Br. Eur., including phytohormone-insensitive types and hormone overproducing strains, has revealed key developmental transitions and clarified the roles of auxins and cytokinins in regulating gametophytic morphogenesis. Also, genetic analysis of some categories of developmental mutants has provided insight into the complexity of certain morphogenetic phenomena. Further advances, including the isolation of developmental genes, identification of their products and mode of action, as well as elucidation of the mechanisms responsible for controlling their expression, will require utilization of the tools of modern molecular genetics. Almost certainly, the establishment of reliable methods for genetically transforming *P. patens* will be central to these efforts. Here, the generation of stably transformed *P. patens* by the introduction into its cells of a plasmid, pBI426, is described. This plasmid possesses two marker genes, engineered for expression in eukaryotic cells, which facilitate the selection and recognition of moss transformants. Preliminary analysis of the transformants suggests that, in most cases, the introduced plasmid is not integrated into the moss's genome; instead it appears to remain free and functions as an autonomous replicon. The application of a novel approach, computer-modelling with Lindenmayer systems (L-systems), has allowed additional insight into the nature and complexity of *P. patens* morphogenesis at the cell and tissue levels and is described briefly.

Key words: *Physcomitrella*; moss; mutants; phytohormones; development; genetic transformation; computer-modelling.

Ultimately, the processes underlying cell differentiation and morphogenesis during development of a moss spore into a complete gametophyte will be elucidated at the cellular, molecular and genetic levels. The isolation and study of developmental mutants of *Physcomitrella patens* by conventional mutagenic procedures (Ashton, Cove and Featherstone 1979; Ashton, Cove, Wang and Saunders 1988; Cove and Ashton 1988; Knight, Cove, Boyd and Ashton 1988; Ashton and Cove 1990) have helped to reveal key developmental transitions and have led to a better appreciation of the roles of hormones, namely auxins and cytokinins, in regulating gametophytic morphogenesis (Cove and Ashton 1988; Ashton and Cove 1990). In addition, genetic analysis of some categories of developmental mutants has provided indications of the complexity of certain developmental phenomena (Grimsley, Featherstone, Courtice, Ashton and Cove 1979; Featherstone, Cove and Ashton 1990). More recently, computer-modelling of *P. patens* morphogenesis has contributed to a fuller appreciation of its complexity at the tissue and cell levels. Thus far, isolation of developmental genes, identification of their products and mode of action, as well as regulation of the expression of developmental genes, have remained tantalizingly elusive. If, however, genetic transformation technology were developed to a sufficiently sophisticated level to allow *P. patens* developmental genes to be insertionally inactivated by foreign DNA, for example (insertional mutation), or developmental mutants to be repaired by plasmids bearing wild-type alleles of developmental genes (mutant rescue), this should expedite the realization of the basic goals of developmental biology.

[1] This work has been partially funded by NSERC.

Some recent progress which we and others have made towards achieving these ambitions is described below.

Progress To Date

Analysis of the Complexity of *Physcomitrella patens* Development at the Cell and Tissue Levels.

1.) Isolation of Developmental Mutants and Their Analysis.
We have selectively isolated many mutants of *P. patens* which are resistant to the synthetic auxin, naphth-1ylacetic acid (NAA), (*NAR* strains) or the synthetic cytokinin, 6–benzylaminopurine (BAP), (*BAR* strains). A high proportion of these mutants are developmentally abnormal, exhibiting altered patterns of morphogenesis and cell differentiation. The developmental pattern of some *NAR* mutants is repairable with exogenous cytokinin, and some *BAR* strains are repairable with exogenous auxin; other developmental mutants are insensitive to both hormonal types (Ashton, Grimsley and Cove 1979; Ashton, Cove, Wang and Saunders 1988; Cove and Ashton 1988; Knight, Cove, Boyd and Ashton 1988; Ashton and Cove 1990). Extensive cytological and physiological analyses of *NAR* and *BAR* mutants, as well as several other categories of morphologically-altered strains (Ashton, Cove and Featherstone 1979; Knight, Cove, Boyd and Ashton 1988), have facilitated construction of an elaborate model of the roles of these hormones and some other factors e.g. light, in controlling cell differentiation and morphogenesis in this moss. They have also allowed evaluation of the complexity of gametophyte development (Ashton, Cove and Featherstone 1979; Ashton, Grimsley and Cove 1979; Ashton, Cove, Wang and Saunders 1988; Cove and Ashton 1988; Ashton and Cove 1990). Figure 1 is a schematic representation of this model.

2.) Additional Evidences supporting the view that endogenous auxin(s) and cytokinins are important regulators of growth and development in *P. patens* include:

(a) The extraction, and identification, of endogenous indole-3-acetic acid (IAA) from *P. patens* gametophytes (Ashton, Schulze, Hall and Bandurski 1985).

(b) The observation that strains resistant to NAA are resistant to IAA also; strains repairable with NAA are sensitive to IAA also (Ashton, unpubl. data).

(c) Auxin accumulation studies (Ashton 1987; Ashton, Boyd, Cove and Knight 1990; Ashton and Cove 1990) show that wild type *P. patens* possesses a saturable IAA influx carrier in the plasma membrane. In summary, these investigations, utilizing ^{14}C-IAA and ^{3}H-IAA in both centrifugation and filtration assays, reveal that auxin accumulation by *P. patens* has the following characteristics:

(i) it is abolished by freeze-thawing.
(ii) it is highly pH-dependent, being greatest when the pH of the incubation medium is low and being reduced very markedly by the protonophore, 2, 4-dinitrophenol (DNP).
(iii) it has a saturable component, i.e. the accumulation of labelled IAA is reduced by an excess of unlabelled IAA.
(iv) it is increased by the auxin transport inhibitor, naphth-1ylphthalamic acid (NPA).

These results are consistent with the chemiosmotic model of auxin accumulation and transport (Rubery and Sheldrake 1974) and indicate that, as has been suggested for some higher plant cells, e.g corn coleoptile cells (Sussman and Goldsmith 1981), there may be two kinds of auxin carriers in the plasma membrane (PM): (a) an IAA anion/proton symport responsible for auxin influx. (b) a uniport which effects efflux of IAA anions and which also interacts with auxin transport inhibitors of which NPA is one example.

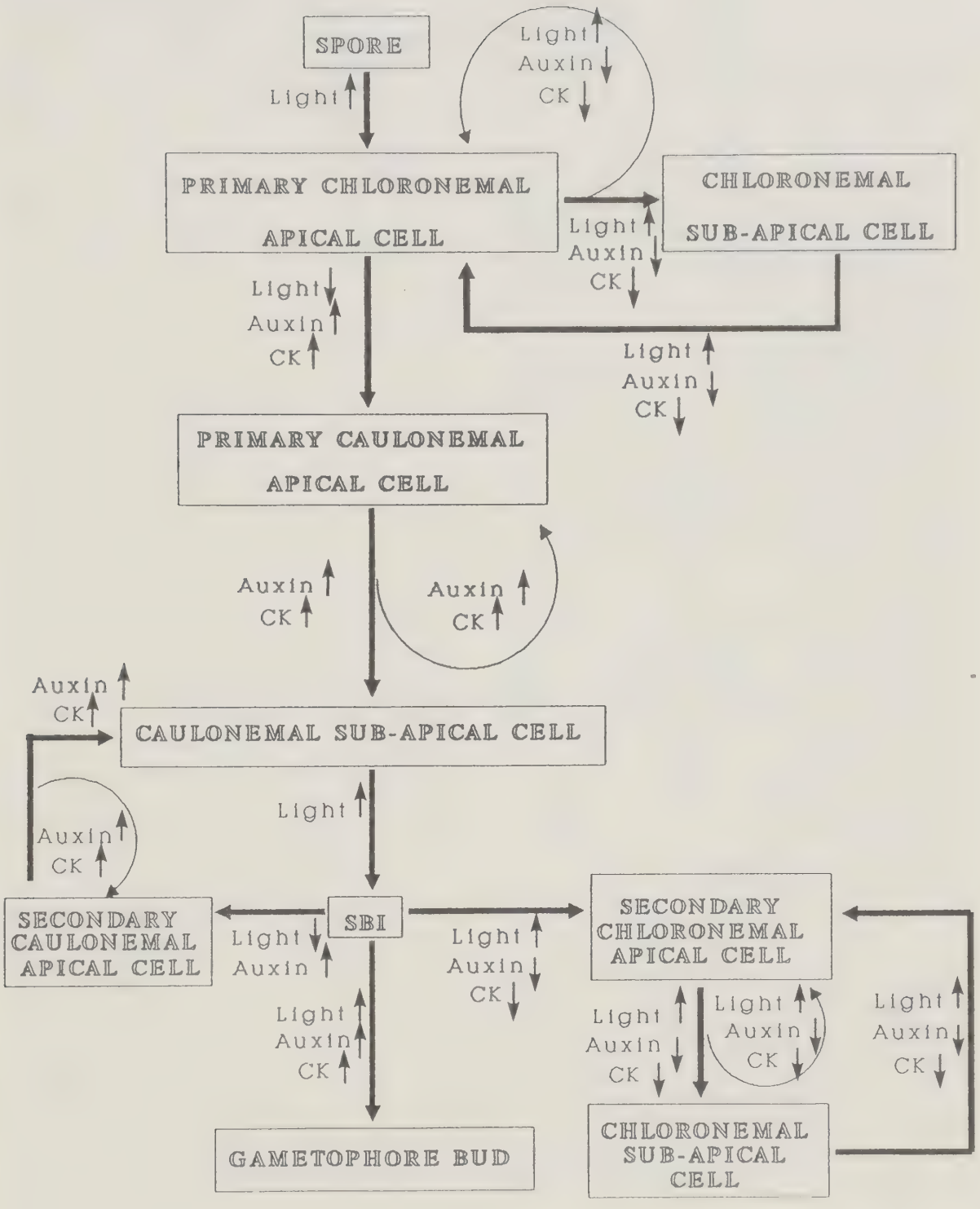

Fig. 1. The roles of the phytohormones, auxin and cytokinin, and their interaction with light in controlling cell differentiation in *P. patens*.
Abbreviations: CK, Cytokinin; SBI, Side Branch Initial.
↑– indicates that the factor promotes the transition.
↓– indicates that the factor inhibits the transition

d) Preliminary auxin binding studies have detected auxin binding sites associated with *P. patens* membranes, especially the endoplasmic reticulum (Reichert, Ashton and Ray 1982).

e) Data corroborating the existence of a PM-located, NPA-sensitive IAA efflux carrier have been obtained by searching for membranous NPA binding sites in wild type *P. patens*. We have detected specific, i.e. displaceable or saturable, binding of ^{3}H-NPA to general membrane preparations (Ashton and Jacobs, unpubl. data). Interestingly, the specifically bound NPA could be completely displaced from the moss membrane with quercetin which belongs to a group of naturally occurring plant flavonols some of which, it has been suggested (Jacobs and Rubery 1988), may function as endogenous auxin transport inhibitors.

(f) Wild type *P. patens* grown on auxin transport inhibitors, including NPA and triiodobenzoic acid, phenocopies some categories of developmentally abnormal *NAR* mutants (Ashton, unpubl. data).

(g) N^6–(Δ^2–isopentenyl) adenine (IPA) and zeatin have been isolated from wild type *P. patens* and gametophore overproducing developmental strains (*OVE* mutants)(Fig. 2) (Wang, Horgan and Cove 1981).

(h) Wild type *P. patens* grown on exogenous cytokinin phenocopies some categories of *OVE* mutants (Ashton, Cove and Featherstone 1979).

3.) Computer-modelling

Using Lindenmayer systems (L-systems) (Lindenmayer 1968) and advanced computer graphics, we have realistically modelled *P. patens* development from a single cell into a complete gametophyte (Fig. 3. Figure 4, a real moss gametophyte, has been included for comparison purposes.) This has been possible mainly for two reasons: (1) The essential nature of L-systems is that they define complex objects by replacing parts of a simple object using a set of rewriting rules; rewriting is performed recursively and in parallel resulting in successively more complex objects. (2) Observations of actual morphogenetic sequences in *P. patens* have provided the information required to define sets of rewriting rules for L-systems. The power of this technique results, in part, from its ability to allow the investigator to readily vary the model rules which are extremely difficult or impossible to change in the real organism. The interactive component of the modelling process generates predictions about plant growth which can be tested by reference to further observations made on the moss as it grows. This novel approach has helped us to recognize key developmental steps and to estimate, from the number of rules needed to model *P. patens*, the level of complexity of the growth and development of this plant. Encouragingly, the number of rules required at the cell and tissue levels is small. The computer simulation of *P. patens* consists of two phases: model generation and model interpretation. Model generation involves the construction of the final complex object from simple objects described by an L-system model. The basic idea is to define a complex object by repeatedly replacing simpler parts of the object. This is done by specifying an initial object (e.g. ***A***) and a set of rules that describe how that object and those that follow will change (e.g. $\boldsymbol{A} \rightarrow \boldsymbol{AB}$, $\boldsymbol{B} \rightarrow \boldsymbol{A}$). Thus, successive developmental stages en route to the final object become ever more complex (e.g. ***A*** becomes ***AB*** which then becomes ***ABA***, then ***ABAAB*** and so on.). Model interpretation is the representation of object parts using various computer graphics techniques.

Analysis of the Complexity of *Physcomitrella patens* Development at the Genetic/Molecular level

Complementation analysis has been used successfully to assess the genetic complexity of various groups of *P. patens* biochemical mutants (Grimsley, Ashton and Cove 1977a, b) and a partial analysis of *GAD*

Fig. 2. The peripheral caulonemata of an *OVE* mutant with many abnormal gametophores.

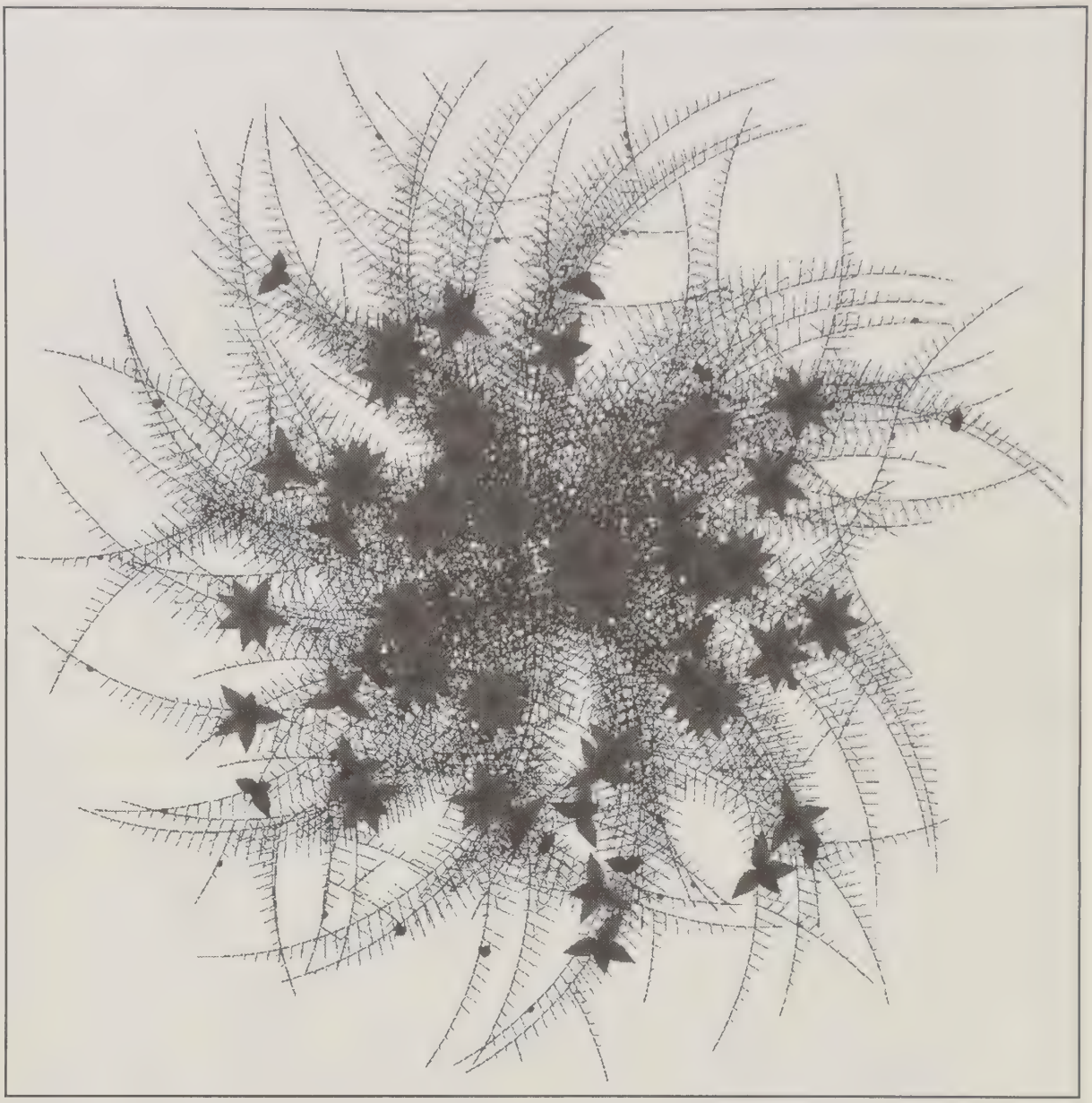

Fig. 3. A computer-generated model of a 16 day old gametophyte of *Physcomitrella patens*.
(See front cover for color version of this figure)

(gametophore developmental) mutants has been achieved (Grimsley, Featherstone, Courtice, Ashton and Cove 1979; Courtice and Cove 1983). Unfortunately, some strains belonging to important developmental mutant categories, including many *NAR* and *NAR/BAR* mutants, contain dominant alleles of developmental genes making these strains refractory to complementation testing (Ashton, Grimsley and Cove 1979; Grimsley, Featherstone, Courtice, Ashton and Cove 1979). However, we and our collaborators have dissected the genetic complexity of one very important group of morphogenetically abnormal mutants, namely category 9 *OVE* strains (Featherstone, Cove and Ashton 1990). These mutants exhibit about a 100-fold increase in gametophore-bud production (Fig. 2) and synthesize approximately 100-times more endogenous cytokinin, including IPA, the nucleoside monophosphate derivative of IPA, and zeatin, than wild type (WT) *P. patens* (Wang, Horgan and Cove 1981). IPA appears to be

Fig. 4. A real 16 day old moss gametophyte

the major cytokinin in *P. patens*, being produced in amounts at least 10-fold greater than those of zeatin in both WT and *OVE* mutants. Genetic analysis by means of somatic hybridization, achieved through protoplast fusion, has revealed that, of 15 independently isolated *OVE* mutants, 14 carry recessive mutations responsible for this abnormal phenotype. Seven of these strains have been assigned to three complementation groups: *OVEA*, *OVEB*, and *OVEC*. A further three strains have been demonstrated not to belong to the *OVEA* group and another mutant does not fall into group *OVEB*. Phenotypic segregation ratios among progeny obtained following self-fertilization of a number of different somatic hybrids showed that several *OVE* mutations behave as recessive alleles of single Mendelian genes (Featherstone, Cove and Ashton 1990).

Significance

The major significance of the progress described above is:

1.) Cytological observation indicates that the moss protonema consists of only two main cell types; mutant analysis and computer-modelling suggest that about 20–30 rules/steps are involved at the cell and tissue levels in the development of a spore into a gametophyte; and complementation analysis of mutant groups affected at a single morphogenetic transition, e.g. *OVE* mutants, reveals that the complexity of each step at the genetic/molecular level may not be extreme. Thus, the elucidation of developmental phenomena in this simple plant model system appears feasible.

2.) Important types of developmental mutants sought by novel non-conventional methods e.g. insertional mutagenesis by transforming DNA, will be readily recognized.

The Genetic Transformation of *P. patens* and Prospects for Future Progress

Rapid advances in comprehending, at the molecular level, mechanisms controlling *P. patens* cell differentiation and morphogenesis, will almost certainly derive from the application and refinement of genetic transformation technology in this moss. Insertional mutagenesis of *P. patens* genes achieved through transformation with appropriate foreign DNA would be an especially powerful tool for the molecular dissection of development, since the inactivated genes would be identifiable, flanking the transforming DNA on both sides. Isolation, cloning, sequencing, and further analysis of the developmental genes affected would then be feasible.

Schaefer and co-workers have reported significant progress towards these ends. They have successfully transformed *P. patens* with a variety of plasmids carrying selectable markers (Schaefer, Zryd, Knight and Cove 1991). They have also provided evidence that in some cases the transforming DNA has been stably integrated into the moss's genome. Although no developmental mutants were generated by insertional inactivation, the work constitutes significant progress towards this end.

Using a modified version of Schaefer's method together with the plasmid, pBI426 (a gift from Dr. W.L. Crosby), a pUC19 derivative containing a GUS::NPT-II transcriptional and translational fusion gene (Datla, Hammerlindl, Pelcher, Crosby and Selvaraj 1991) flanked upstream by two tandem Cauliflower Mosaic Virus 35S promoters and downstream by a *nos* transcriptional terminator, we have isolated approximately 1000 transformants (Zacharias 1991). Approximately one billion regenerating protoplasts were screened for resistance to the kanamycin analogue, G418, at a concentration of 50 $\mu g\ ml^{-1}$. More than 700 of these pBI426 transformants have been subcultured repeatedly onto fresh G418-containing medium and have exhibited no diminution in their resistance to the antibiotic (Zacharias 1991). A minority (<10) of these strains are developmentally abnormal and in a few cases they resemble, morphologically, *NAR/BAR* mutants. That these G418-resistant strains are truly genetic transformants is indicated by the following:

a) They occur at a frequency of about 1 in 50,000 which is very much higher than observed rates of spontaneous mutation to G418 resistance or to any other mutant phenotype.

b) Almost without exception, these strains lose irreversibly the antibiotic resistance trait upon relaxation of selective pressure, i.e. if cultured for a few weeks upon minimal medium (Zacharias 1991; Verkoczy 1992).

c) High levels of ß-glucuronidase (GUS) activity have been observed in each of six transformants chosen at random from the larger sample. GUS activity was detected using the X-GLUC histological method

(Jefferson, Kavanagh and Bevan 1987) and quantified by fluorimetry after provision of MUG as enzyme substrate (Jefferson, Kavanagh and Bevan 1987). GUS was detected in these transformants only when they had been grown in the presence of G418 (Zacharias 1991).

d) Amplified DNA fragments derived from the NPT-II gene of the transforming plasmid have been obtained from 23 of 27 transformed strains tested by the polymerase chain reaction (Verkoczy 1992).

A plausible explanation of the phenotypic characteristics of the majority of these pBI426 transformants is that the plasmid has not been recombined into the moss's genome and that it replicates autonomously but more slowly than the normal rate at which cell division occurs. Thus, while positive selection is maintained, moss cells divide more slowly, plasmid replication keeps pace with cell division and the transformed state is stable. Upon relaxation of selective pressure, cell division outstrips plasmid replication, the plasmid is lost by dilution and the transformed phenotype is irreversibly lost. If this explanation is correct, then the isolation of moss developmental genes will be possible by mutant rescue. In brief, the approach employed will involve transforming developmental mutants with plasmids such as pBI426 into which have been cloned random or defined fragments of WT *P. patens* genomic DNA. Any transformants generated will be screened for those in which the mutant developmental phenotype has been repaired to WT. Such an occurrence may result in some cases from homologous recombination between the mutant and WT alleles of the moss gene or, in the absence of recombination, be a consequence of dominance of the WT plasmid-borne allele over its mutant chromosomal counterpart. Should the latter be true, extraction of the transforming plasmid carrying the WT developmental gene would be relatively facile.

It is clear from the above discussion that elaboration of more precise strategies for using genetic transformation to dissect developmental phenomena in *P. patens* depends, in the first instance, upon ascertaining the exact nature of the transformation events already observed. Therefore, questions which are currently being addressed as a matter of urgency, primarily through Southern hybridization experiments, revolve around whether or not the transforming plasmid has been integrated into the host's genome: if integrated, whether one or several copies are present, whether recombination has occurred at a single or several sites and whether such sites are distributed randomly in the moss's genome. Although it seems likely that both integrative and non-integrative (replicative) transformation can occur in *P. patens*, probably most, if not all, of the pBI426-transformed strains referred to in this paper have arisen from the latter type of transformation event.

Conclusion

Recently, *Physcomitrella patens* has become quite popular as a simple model system for studying plant development. Reasons for this include:

1.) The occurrence in *P. patens* of developmental phenomena analogous to those which can be observed in higher plants.

2.) The possession of identical or similar phytohormones to those present in higher plants and their involvement in the moss as important regulators of cell differentiation and morphogenesis.

3.) Its ease of axenic culture on simple, defined media.

4.) Its small size and short life cycle.

5.) Its small number of cell types.

6.) The extended haploid gametophytic phase of its life cycle which facilitates the isolation of a wide variety of mutants includ-

ing morphological, phytohormone-insensitive and phytohormone-requiring types.

7.) The existence of established methodologies for genetic analysis.

The development and continued refinement of genetic transformation methods and also of computer-modelling techniques for use with this bryophyte should further enhance its usefulness as a plant model system.

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Isozyme evidence proves that the moss *Rhizomnium pseudopunctatum* is an allopolyploid of *R. gracile* x *R. magnifolium* [1]

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WYATT, R., I. J. ODRZYKOSKI, and A. STONEBURNER (Department of Botany, University of Georgia, Athens, GA 30602). Isozyme evidence proves that the moss *Rhizomnium pseudopunctatum* is an allopolyploid of *R. gracile* x *R. magnifolium*. Mem. Torrey Bot. Club 25(1): 21–35. 1993. – Genetic markers from horizontal starch-gel electrophoresis indicate that *Rhizomnium pseudopunctatum* is an allopolyploid whose haploid progenitors were genetically very similar to present-day *R. gracile* and *R. magnifolium*. Because the progenitors are genetically very distinct (mean genetic identity, I = 0.687), *R. pseudopunctatum* displays fixed heterozygosity at about one-third of the 16 loci surveyed. Remarkably low levels of genetic polymorphism in *R. gracile* and *R. magnifolium* preclude any certainty on the issue of possible multiple origins of *R. pseudopunctatum*. We detected three alleles unique to the allopolyploid, only one of which occurred in high enough frequency and in sufficient numbers of geographically separated populations that it could not be explained simply as a product of post-hybridization mutation. Only one locus in the polyploid showed any possible evidence of silencing of duplicated genes. It therefore appears likely that *R. pseudopunctatum* originated very recently, perhaps during the Pleistocene, when the ranges of its boreal progenitors were displaced to the south. Moreover, bottlenecks in population size that occurred in association with this southward migration may explain the low levels of genetic variation in *R. gracile* and *R. magnifolium*. Finally, it appears that the striking lack of genetic differentiation among populations of these mosses, even between intercontinentally disjunct sites, is due to recent recolonization of deglaciated areas only 5-8 x 10^4 years B. P. *Rhizomnium pseudopunctatum* joins *Plagiomnium medium* as the only documented allopolyploid mosses, further substantiating that reticulate evolution is possible in bryophytes.

Key words: Moss, polyploidy, *Rhizomnium*, isozymes, Mniaceae, genetic variation, hybridization.

Nearly 45 years ago, Lowry (1948) published a now-classic cytotaxonomic study of certain mosses of the family Mniaceae. On the basis of chromosome numbers and morphology, he proposed a series of "species pairs," consisting of a haploid progenitor and a derived autodiploid (see Wyatt 1985 for an updated list of these taxa). In some cases, his choices of progenitor taxa have been questioned and alternative progenitors proposed (e.g., Mogensen 1973, Koponen and Nilson 1977). In the one species pair examined in detail so far, it was discovered that *Plagiomnium medium* (Bruch & Schimp. *in* B. S. G.) T. Kop. subsp. *medium* is an allopolyploid. One of its parents is *P. ellipticum* (Brid.) T. Kop., as predicted by Lowry (1948), whereas its other parent is *P. insigne* (Mitt.) T. Kop. (Wyatt *et al.* 1988, 1992), as predicted by Koponen (1971).

Rhizomnium section *Rhizomnium* includes one diploid [*R. pseudopunctatum* (Bruch & Schimp. in B. S. G.) T. Kop.] and 12 haploid taxa. Lowry (1948) proposed *R. punctatum* [including in the broad sense of Andrews (1940): *R. appalachianum* T. Kop., *R. magnifolium* (Horik.) T. Kop., *R. punctatum* (Hedw.) T. Kop. subsp. *punctatum*, and *R.*

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punctatum subsp. *chlorophyllosum* (Kindb.) T. Kop.] as the most likely progenitor of autopolyploid *R. pseudopunctatum*. In contrast, Koponen and Nilsson (1977) argued strongly that *R. gracile* T. Kop. is "the most probable, and from the morphological point of view the only possible, candidate." They also pointed out that Brassard (1974) was in error in citing Koponen's (1973) work as suggesting that *R. andrewsianum* (Steere) T. Kop. is the progenitor of *R. pseudopunctatum*. Wyatt's (1985) examination of the voucher specimen cited by Lowry (1948) as "typical, dioicous *Mnium punctatum*" revealed it to be a mixed collection, consisting mostly of *R. punctatum* subsp. *chlorophyllosum* but containing a few shoots of *R. magnifolium*. It is most likely, therefore, that Lowry's (1948) chromosome counts were of *R. punctatum* subsp. *chlorophyllosum* and that he considered this taxon the most likely progenitor of *R. pseudopunctatum*.

We decided to use isozymes to determine the nature of polyploidy in *R. pseudopunctatum* and to identify its haploid progenitor(s). This approach has proven useful in analyzing similar situations in other groups of plants, including mosses (Wyatt *et al.* 1988, 1992), pteridophytes (Werth 1989), and angiosperms (Crawford 1985; Weeden and Wendel 1989). Allopolyploids typically express fixed heterozygous phenotypes at loci that differ electrophoretically in the progenitor species (e.g., Roose and Gottlieb 1976; Werth *et al.* 1985a, 1985b), whereas autopolyploids usually show polysomic inheritance and variable expression including unbalanced heterozygotes (e.g., Soltis and Rieseberg 1986; Soltis and Soltis 1988, 1989). Elsewhere we will report on our more extensive studies of eight of the 13 taxa of *Rhizomnium* section *Rhizomnium*, which clearly eliminate all but two taxa as possible progenitors (Wyatt *et al.*, unpublished). Here we report on our sampling of populations of *Rhizomnium pseudopunctatum, R. gracile,* and *R. magnifolium* from throughout their respective ranges.

Materials and Methods

Population Samples

We sampled a total of 23 populations: 11 of *R. magnifolium*, 7 of *R. pseudopunctatum*, and 5 of *R. gracile*. Populations of *R. magnifolium* came from California, Oregon, and Washington in the United States and from Sweden, Norway, and Finland. Those of *R. pseudopunctatum* came from northern Ontario, Canada (Stoneburner and Wyatt 1991), and from Sweden, Norway, and Finland. Material of *R. gracile* came from northern Ontario and Alaska (Wyatt and Stoneburner 1991).

At each site we collected approximately 5-cm x 5-cm clumps from within discrete colonies spaced at least 1 m apart, placing these samples into plastic bags, which were stored at 4°C. We usually collected along streams or the edges of wetlands until we had sampled a total of 48 discrete colonies. This typically entailed covering a linear distance of about 0.5–1.0 km. Species identities were verified in the laboratory under a dissecting microscope, and single, healthy shoots were selected for enzyme extraction. In addition, we made chromosome counts from mitotic figures in shoot tips for at least two plants from each population.

Electrophoretic Procedures

Our procedures for horizontal starch-gel electrophoresis were similar to those described in our earlier work on *Plagiomnium ciliare* (Wyatt *et al.* 1989a) and other members of section *Rosulata* (Wyatt *et al.* 1992). Single moss shoots were homogenized in 50–100 µl of extraction buffer [0.1 M tris HCL, pH 7.5, containing 10 mM KCl, 10 mM $MgCl_2 \cdot 6H_20$, 1 mM EDTA (Na_2 salt), 0.1% Triton X-100, and 42 mM 2- mercaptoethanol]. The extract was filtered through a small strip of Miracloth onto 4-mm x 8-mm Whatman 3MM paper wicks. All of these steps were done over crushed ice. Wicks were stored in an ultracold freezer at –70°C.

Saturated wicks were placed into a vertical slot (the origin) cut across a 10.5% starch gel (Sigma electrostarch), and

enzymes were separated in one of two buffer systems. A modified discontinuous Poulik buffer (PK) was used to resolve NADP-dependent malate dehydrogenase (= malic enzyme, ME: E.C. 1.1.1.40), glucose-6-phosphate isomerase (= phosphoglucoisomerase, PGI: E.C.5.3.1.9), triose-phosphate isomerase (TPI: E.C.5.3.1.1), aspartate aminotransferase (= glutamate oxaloacetate transaminase, GOT: E.C.2.6.1.1), mannose phosphate isomerase (MPI: E.C.5.3.1.8), hexokinase (HEX: E.C.2.7.1.1), fluorescent esterase (FLE: E.C.3.1.1.-), fructose-bisphosphate aldolase (ALD: E.C.4.1.2.13), and peroxidase (PER: E.C.1.11.1.7). The electrode buffer consisted of 300 mM boric acid and 100 mM NaOH at pH 8.6. The gel buffer for this system was 30 mM Tris and 7 mM citric acid at pH 7.6. A modified continuous histidine-citrate buffer (HC), which resolved aconitate hydratase (= aconitase, ACO: E.C.4.2.1.3), NAD-dependent malate dehydrogenase (MDH: E.C.1.1.1.37), glyceraldehyde-3-phosphate dehydrogenase (G3P: E.C.1.2.1.12), phosphogluconcate dehydrogenase (PGD: E.C.1.1.1.44), and phosphoglucomutase (PGM: E.C. 5.4.2.2), consisted of an electrode buffer with 125 mM Tris and 72 mM citric acid at pH 7.0. The gel buffer was 3.5 mM DL-histidine HCL titrated to pH 7.0 with 0.1 M NaOH.

Gels were run in an upright, refrigerated chamber at 4°C. PK gels were run for 15 min at 50 mA and then adjusted to a constant 200 V for 4 hr. HC gels were run for 15 min at 20 mA (ca. 110 V) and then at 25 mA (ca. 150 V) for 4 hr. After separation, enzymes were visualized using standard colorimetric staining methods (Vallejos 1983; Wendel and Weeden 1989). Except for FLE, GOT, and PER, which were stained for 1-3 hr in liquid assay, all enzymes were stained for 1–3 hr using the agar-overlay method. Staining was done in an incubator at 37°C.

All isozymes migrated anodally, and putative loci were numbered in order of decreasing mobility (i.e., fastest = 1). Alleles were also assigned letters in decreasing order of mobility (i.e., fastest = a). To facilitate comparisons across species, in this paper we use allele designations that apply to all eight species of *Rhizomnium* that we have surveyed. Allele frequencies for the 16 putative loci were tabulated and analyzed using the PC versions of BIOSYS –1 (Swofford and Selander 1981) and LYNSPROG, a program developed by Drs. M. D. Loveless and A. F. Schnabel. In the analysis of gene diversity within and among populations, monomorphic loci were omitted.

As Wyatt *et al.* (1992) have pointed out, comparisons of levels of genetic polymorphism are complicated by the inclusion of taxa of different ploidy levels. Usual measures, such as percentage of polymorphic loci, number of alleles per locus, and expected heterozygosity are misleading, because much of the apparent variation in allopolyploids is actually present as fixed heterozygosity. This variation does not segregate at individual gene loci, although it can be recombined across loci. Therefore, our consideration of genetic variation and its partitioning within species will be largely restricted to *R. magnifolium* and *R. gracile*, the haploid taxa.

Results

Electrophoretic Patterns

All of the enzymes we screened expressed subunit structures consistent with Weeden and Wendel's (1989) summary of the expected number of subunits. Moreover, except for TPI, the number of isozymes expressed never exceeded the number expected under Gottlieb's (1982) model. Below we describe our interpretations of the patterns observed for each enzyme in the three species. A more complete discussion of these patterns in relation to all of the taxa we have analyzed in *Rhizomnium* section *Rhizomnium* will be published elsewhere.

ACO: One zone of activity was strongly expressed in all three species. Single bands were always observed for the haploids, *R. gracile* and *R. magnifolium*. Most plants of the polyploid, *R. pseudopunctatum*, showed a fixed two-banded phenotype. Some plants, however, displayed a one-banded pheno-

type that resembled that of *R. gracile*. In addition, plants of *R. pseudopunctatum* sometimes weakly expressed a second, slower isozyme of aconitase. It appeared to be two-banded but was not consistently scorable.

ALD: We observed and scored a single zone of activity, for which plants of all three species proved monomorphic.

FLE: A single zone of strong activity was observed and scored. Although differences were detected in other species of *Rhizomnium* (Wyatt *et al.*, unpublished), these three species were identical in expressing a single, invariant band. One plant of *R. gracile* from a population in northern Ontario did express a band of slightly slower mobility.

G3P: Plants of all three species showed identical phenotypes, consisting of a single, invariant band.

GOT: A single zone of activity was strongly expressed. No variation was detected in *R. gracile*, but two populations of *R. magnifolium* expressed a second allele, and one population of *R. pseudopunctatum* contained four plants that exhibited heterozygous phenotypes. The source of the Got-1c allele is problematical, however, as it was not detected in populations of either haploid progenitor.

HEX: Two zones of activity were observed, but only the faster migrating set of bands could be scored consistently. All three species were monomorphic.

MDH: A single zone of strong activity was scored. Phenotypes for both *R. gracile* and *R. magnifolium* were identical, being three-banded and showing no variation across populations. The polyploid *R. pseudopunctatum*, however, included some plants in each population with four-banded (apparent fixed-heterozygous) phenotypes. Each population also contained some plants with simplified, three-banded patterns that appeared identical to those of *R. gracile* and *R. magnifolium*.

ME: We detected and scored only one zone of activity. Plants of *R. magnifolium* expressed a single, very dark band (Fig. 1). On the other hand, *R. gracile* expressed a four-banded phenotype. The phenotype of *R. pseudopunctatum* appeared to combine these variants into a complex, five-banded pattern. None of the plants in any of the populations of all three species deviated from this pattern.

MPI: The single zone of activity was scored and proved to be invariant across all populations of all three species.

PER: Our staining procedures allowed us to score a single, very fast zone of activity. The single-banded phenotypes for which the haploid progenitors were fixed were combined in the fixed two-banded pattern of *R. pseudopunctatum*.

PGD: The single zone of activity was scored and proved to be invariant across all populations of all three species.

PGI: We detected two zones of activity, but only the slower zone was consistently scoreable. Limited variation was seen in both *R. gracile* and *R. magnifolium*, but *R. pseudopunctatum* consistently expressed a fixed heterozygous phenotype. As expected, this three-banded pattern combined the bands of the haploid progenitors, with strongest expression of the heterodimeric band (Fig. 2).

PGM: We were able to score two zones of activity, the faster of which was invariant in *R. gracile* and *R. magnifolium*. A few plants in two populations of *R. pseudopunctatum* from northern Ontario showed two-banded phenotypes that combined the Pgm-1c allele with the Pgm-1b allele. Otherwise, all plants in all populations expressed the same pattern, apparently being fixed for the Pgm-1b allele.

TPI: Isozyme patterns were very complex, typically consisting of seven bands of varying intensity (Fig. 3). The patterns probably represent the products of four loci and some of the heterodimeric combinations among them. Taking a conservative approach, we scored only the fastest and slowest zones of activity, which proved to be monomorphic across nearly all populations of all three species. The one exception was a population of *R. magnifolium* from a

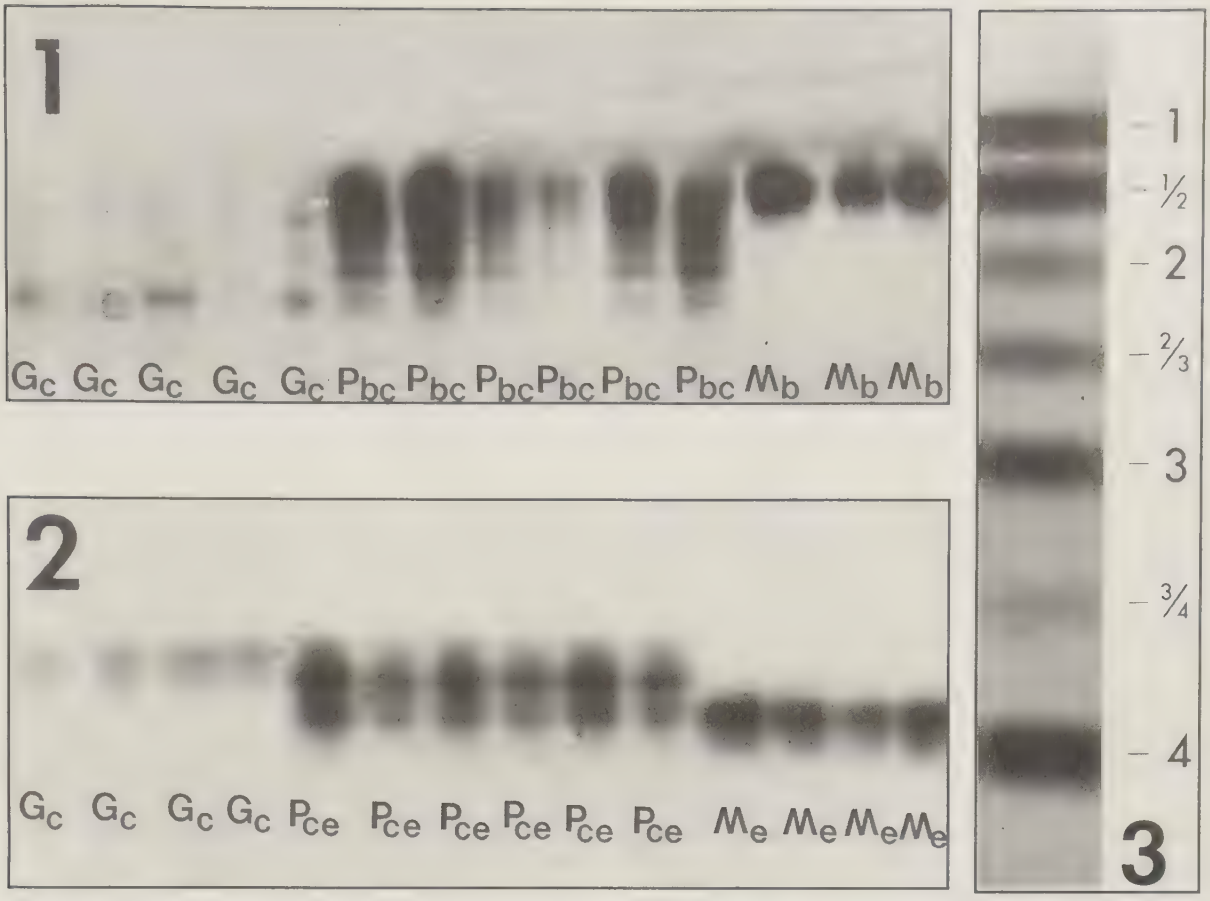

Fig. 1. Photograph of a starch gel illustrating electrophoretic banding patterns of individual gametophytic plants of the allopolyploid moss *Rhizomnium pseudopunctatum* (P) and its two haploid progenitors, *R. gracile* (G) and *R. magnifolium* (M), for the Me-1 isozyme. Subscripts to species designations indicate allelic combinations. Note that the four-banded Me-1c, which is fixed in *R. gracile*, is combined with the single-banded Me-1b of *R. magnifolium* to give a fixed heterozygous, five-banded pattern in *R. pseudopunctatum* (as expected of a tetrameric enzyme).

Fig. 2. Photograph of a starch gel illustrating electrophoretic banding patterns of individual gametophytic plants of the allopolyploid moss *Rhizomnium pseudopunctatum* (P) and its two haploid progenitors, *R. gracile* (G) and *R. magnifolium* (M), for the Pgi-2 isozyme. Subscripts to species designations indicate allelic combinations. Note that the Pgi-2c allele, which is characteristic of *R. gracile*, is combined with the Pgi-2e allele of *R. magnifolium* to give a fixed heterozygous, three-banded pattern in *R. pseudopunctatum* (as expected of a dimeric enzyme).

Fig. 3. Photograph of one lane of a starch gel illustrating the electrophoretic banding pattern of an individual gametophytic plant of *Rhizomnium magnifolium*. Note the complex seven-banded pattern which apparently results from duplications of both isozymes for TPI. Tentative identifications of the sources of these bands are marked, but note that we scored only Tpi-1 and Tpi-4 in our studies.

site in Oregon, in which the unique Tpi-1d allele occurred at a frequency > 20%. Comparisons of TPI patterns in other species and the discovery of more variation may enable us to determine more definitively the correct interpretation of these complex phenotypes. Both isozymes of TPI are also duplicated in *Plagiomnium* section *Rosulata*, and Wyatt *et al.* (1988, 1989b) have provided photographs of these patterns.

Evidence for Allopolyploidy

Allele frequencies for the 23 populations of *R. gracile*, *R. magnifolium*, and *R. pseudopunctatum* are given in the Appendix. When these frequencies are summed across populations (Table 1), the hybrid nature of the polyploid *R. pseudopunctatum* is obvious. Some loci are perfectly diagnostic: Me-1b, for example, is fixed in *R. magnifolium*, whereas Me-lc is fixed in *R. gracile*. Plants of

Table 1. Sample sizes and allele frequencies pooled across populations for 16 putative gene loci of *Rhizomnium gracile*, *R. pseudopunctatum*, and *R. magnifolium*. Abbreviations for enzyme loci are given in the text. Arrows designate the presumed source of particular alleles. Asterisks denote apparent orphan alleles. Note that allele frequencies for the allopolyploid *R. pseudopunctatum* do not represent segregating polymorphisms, but rather alleles present in fixed heterozygous genotypes.

Locus	Allele	R. gracile	R. pseudopunctatum	R. magnifolium
Aco-1		104	574	638
	a	——	0.294 <——	1.000
	b	1.000 ——>	0.706	——
Ald-1		24	36	18
	a	1.000	1.000	1.000
Fle-1		199	444	128
	a	0.995	1.000	1.000
	b	0.005	——	——
G3p-1		24	36	18
	a	1.000	1.000	1.000
Got-1		191	650	654
	a	——	——	0.051
	b	1.000	0.994	0.949
	c	——	0.006*	——
Hex-1		199	650	661
	a	1.000	1.000	1.000
Mdh-1		193	650	659
	a	——	0.234*	——
	b	1.000	0.766	1.000
Me-1		199	650	661
	b	——	0.500 <——	1.000
	c	1.000 ——>	0.500	——
Mpi-1		199	444	110
	a	1.000	1.000	1.000
Per-1		185	614	640
	a	——	0.500 <——	1.000
	c	1.000 ——>	0.500	——
Pgd-1		24	20	10
	a	1.000	1.000	1.000
Pgi-2		201	650	383
	b	0.035	——	——
	c	0.965 ——>	0.500	——
	e	——	0.500 <——	0.997
	f	——	——	0.003
Pgm-1		199	650	653
	b	1.000	0.980	1.000
	c	——	0.020*	——
Pgm-2		199	650	383
	a	——	0.500 <——	1.000
	b	1.000 ——>	0.500	——
Tpi-1		199	650	655
	c	1.000	1.000	0.979
	d	——	——	0.021
Tpi-4		193	650	655
	d	1.000	1.000	1.000

the allopolyploid *R. pseudopunctatum* invariably express a fixed heterozygous combination of these two alleles (Fig. 1). Per-1 is similar, in that all plants of *R. pseudopunctatum* are fixed heterozygotes for the a allele of *R. magnifolium* and the b allele of *R. gracile*. Pgm-2a is fixed in *R. magnifolium*, whereas *R. gracile* is fixed for Pgm-2b. As expected, all plants of *R. pseudopunctatum* show fixed heterozygous expression of these two alleles.

Another locus contributing information about the nature of *R. pseudopunctatum* as an allopolyploid is Pgi-2. Pgi-2c, which occurs at a frequency of 96.5% in populations of *R. gracile* is combined in *R. pseudopunctatum* with Pgi-2e, which occurs at a frequency of 99.7% in *R. magnifolium*. Given the low frequency and limited geographical distribution of Pgi-2b and Pgi-2f (which occur in *R. gracile* and *R. magnifolium*, respectively), it is perhaps not surprising that neither allele was apparently incorporated into the genome of *R. pseudopunctatum*. Aco-1 also provides evidence of the origin of *R. pseudopunctatum*: many plants are fixed heterozygotes for Aco-1a, which is fixed in *R. magnifolium*, and Aco-1b, which is fixed in *R. gracile*. It is problematical, however, to explain those plants of *R. pseudopunctatum* that express a simplified pattern like that of *R. gracile*, appearing to express only Aco-1b. It is possible that such plants actually possess two Aco-1b alleles, one originating from *R. magnifolium* and the second from *R. gracile*. This interpretation supposes that we simply have not yet sampled enough populations of *R. magnifolium* yet to discover the true overall frequency of alleles (or that the Aco-1b allele has been lost from all present-day populations of *R. magnifolium*). Alternatively, there may have been gene silencing in some populations of allopolyploid *R. pseudopunctatum*, leading to loss of expression of the Aco-1a allele from *R. magnifolium*.

A number of additional loci attest to the close relationship among the three species, as they share alleles. For example, Ald-1, G3p-1, Hex-1, Mpi-1, Pgd-1, and Tpi-4 are monomorphic across all three species. Other loci that show minor variation in *R. gracile* or *R. magnifolium*, but otherwise are nearly identical across species, are Fle-1 and Tpi-1. Populations of the allopolyploid *R. pseudopunctatum* harbor only three orphan alleles (i.e., alleles detected in *R. pseudopunctatum* that were not also detected in either *R. gracile* or *R. magnifolium*). One of these, Got-1c, occurred in only one heterozygous plant from Kongsvold, Norway. It likely represents a recent mutation. Another, Pgm-1c, was expressed by about a dozen heterozygous plants in each of two populations from northern Ontario. These plants also could represent carriers of a post-hybridization mutation. Alternatively, the Pgm-1c allele may have once occurred in populations of *R. gracile* or *R. magnifolium*. The third orphan allele, Mdh-1a, poses more of a problem, as it occurs in frequencies ranging from 4–50% in all but one population (from southern Sweden) of *R. pseudopunctatum*. It seems likely that this allele once occurred in reasonably high frequencies in the populations of *R. gracile* or *R. magnifolium* that hybridized to launch *R. pseudopunctatum* as a new species.

The relatively large genetic distance between *R. gracile* and *R. magnifolium* suggests that these closely related species are unusually distinct, with a mean $\overline{D} = 0.375$ (Table 2). Both are genetically more similar to *R. pseudopunctatum*, with *R. gracile* at $\overline{D} = 0.076$ appearing slightly less distant than *R. magnifolium* at $\overline{D} = 0.109$. According to Werth's (1989) formula, species as distinct as *R. gracile* and *R. magnifolium* should result in an allopolyploid showing fixed heterozygosity at 31.3% of its loci. In fact, we observed fixed heterozygous patterns at 5 of 16 (31.3%) loci in *R. pseudopunctatum*.

Genetic Variation Within Species

Of the 16 loci surveyed for genetic variation, six (Ald-1, G3p-1, Hex-1, Mpi-1, Pgd-1, and Tpi-4) were monomorphic in all populations of all three species. In fact, levels of genetic variation in the haploids, *R. gracile*

Table 2. Means for Nei's (1972) genetic distances (below the diagonal) and Rogers' (1972) genetic similarities (above the diagonal) for populations of *Rhizomnium gracile* (N = 5), *R. pseudopunctatum* (N = 7), and *R. magnifolium* (N = 11) based on 11 putative gene loci scored in all populations. Values on the diagonal are means (and ranges) for Nei's (1972) genetic identity for populations within species.

	R. gracile	R. pseudopunctatum	R. magnifolium
R. gracile	1.000 (1.000–1.000)	0.841	0.684
R. pseudopunctatum	0.076	0.986 (0.965–1.000)	0.811
R. magnifolium	0.375	0.109	0.980 (0.920–1.000)

Table 3. Sample sizes (N), average percentages of polymorphic loci (PLP), mean number of alleles per locus (k), gene diversities (H_S), and standard errors (SE) for 11 enzyme loci surveyed in 23 populations of *Rhizomnium gracile* (1–5), *R. pseudopunctatum* (6–12), and *R. magnifolium* (13–23). Note that N is equal to the number of haploid genomes screened and that estimates of genetic polymorphism are inflated by fixed heterozygosity in the allopolyploid *R. pseudopunctatum*.

Population/Locality	N	PLP	k	Hs ± SE
1 Pisew Road, Moosonee, Ontario, Canada	48	0.0	1.18	0.007 ± 0.005
2 Moose Factory Island, Ontario, Canada	45	9.1	1.09	0.011 ± 0.011
3 Tacan Road, Moosonee, Ontario, Canada	48	9.1	1.09	0.011 ± 0.011
4 Ontario Northland Railway, Moosonee, Ontario, Canada	44	0.0	1.00	0.000 ± 0.000
5 Toolik Lake, AK	14	0.0	1.00	0.000 ± 0.000
6 Måryd, Scania, Sweden	96	33.3	1.33	0.168 ± 0.084
7 Hamptgärn Lake, Umeå, Sweden	110	44.4	1.44	0.214 ± 0.085
8 Kongsvold, Norway	90	44.4	1.56	0.180 ± 0.074
9 Lohja Parish, Finland	94	33.3	1.44	0.158 ± 0.075
10 Hattula, Takajärvi, Taipaleensuo, Finland	96	44.4	1.44	0.216 ± 0.086
11 Pisew Road, Moosonee, Ontario, Canada	96	55.6	1.56	0.192 ± 0.072
12 Pisew Road, Moosonee, Ontario, Canada	68	55.6	1.56	0.206 ± 0.074
13 Snoqualmie Pass, WA	64	0.0	1.00	0.000 ± 0.000
14 Kings Creek, Lassen Volcanic National Park, CA	84	0.0	1.00	0.000 ± 0.000
15 Willamette National Forest, OR	72	11.1	1.11	0.020 ± 0.020
16 Craig Lake, OR	96	11.1	1.11	0.045 ± 0.045
17 Andrews Experimental Forest, OR	24	0.0	1.00	0.000 ± 0.000
18 Umpqua National Forest, OR	74	11.1	1.11	0.037 ± 0.037
19 Umeå, Sweden	45	0.0	1.00	0.000 ± 0.000
20 Umeå, Sweden	92	0.0	1.00	0.000 ± 0.000
21 Kongsvold, Norway	46	0.0	1.00	0.000 ± 0.000
22 Kongsvold, Norway	17	0.0	1.00	0.000 ± 0.000
23 Lammi Biological Station, Finland	47	0.0	1.00	0.000 ± 0.000

Table 4. Means for total gene diversity (H_T) and gene diversities within (H_S) and between (D_{ST}) populations of *Rhizomnium gracile*, *R. magnifolium*, and *R. pseudopunctatum* based on polymorphic loci. Also shown are values for between population diversity relative to total diversity (G_{ST}).

Species	H_T	H_S	D_{ST}	G_{ST}
R. gracile	0.0386	0.0378	0.0009	0.0242
R. magnifolium	0.0476	0.0385	0.0091	0.1492
R. pseudopunctatum	0.3532	0.3403	0.0128	0.0452

and *R. magnifolium*, is remarkably low (Table 3). Using a 5% frequency criterion, none of the 16 loci surveyed in *R. gracile* is polymorphic. A single plant in one population expressed Fle-1b, an allele unique to *R. gracile*. A second unique allele, Pgi-2b occurred in low frequency in three populations from northern Ontario. The expected mean heterozygosity for *R. gracile* was 0.005 ± 0.004 (mean±standard error). *Rhizomnium magnifolium* was only slightly more variable. There was no polymorphism detected in European populations, but populations from North America were polymorphic for Got-1 and expressed unique alleles at Tpi-1 and Pgi-2. For the species as a whole, expected heterozygosity was 0.009 ± 0.006. In contrast, *R. pseudopunctatum* was polymorphic at 50% of its loci, expressed 1.5 ±0.1 alleles per locus, and had expected heterozygosity of 0.177 ± 0.058. Bear in mind, however, that *R. pseudopunctatum* is an allopolyploid and, therefore, much of its apparent variation is fixed because of nonsegregation of nonhomologous chromosomes.

Total gene diversity, based on 11 loci scored in all populations, was 0.0048 for *R. gracile*. For *R. magnifolium*, $H_T = 0.0089$, and for *R. pseudopunctatum*, $H_T = 0.1766$. Considering only polymorphic loci, it is apparent that most of the variation occurs within local populations of all three species (Table 4). G_{ST} values are remarkably low, especially for *R. gracile* and *R. pseudopunctatum*. Only about 3–5% of the genetic variation in these wide-ranging, boreal species is expressed as differences among populations.

Within all three species, Nei's (1972) genetic identities between populations are very high, with all $\bar{I} > 0.98$. This is true despite the extremely wide range over which populations of the circumboreal *R. magnifolium* and *R. pseudopunctatum* were sampled. A UPGMA phenogram summarizing Rogers' (1972) genetic similarities among populations reinforces the view that there is little differentiation among populations and that the differentiation that does exist fails to parallel geographical disjunctions (Fig. 4). In *R. gracile*, one population from northern Ontario links up with a population from Alaska, more than 4,000 km away, before it links up with other Canadian populations. In *R. magnifolium* there are genetically identical populations separated by nearly 10,000 km! Similarly, some Scandinavian populations of *R. pseudopunctatum* are more genetically similar to Canadian populations on the other side of the Atlantic Ocean than they are to other populations from Sweden and Finland.

Another striking result of the comparisons of genetic similarity is the relatively small degrees of differentiation within, as opposed to between, species (Fig. 4). Within species the lowest genetic similarities were still > 0.90, whereas values were often < 0.70 between species.

Chromosome Counts

All counts obtained from populations of *R. magnifolium* and *R. gracile* were $n = 7$, consistent with their behavior as isozymic haploids. Conversely, all counts of *R. pseudopunctatum* were approximately $n = 14$, again agreeing with the observation of diploid patterns at many loci. Although it was always clear that the number of chromo-

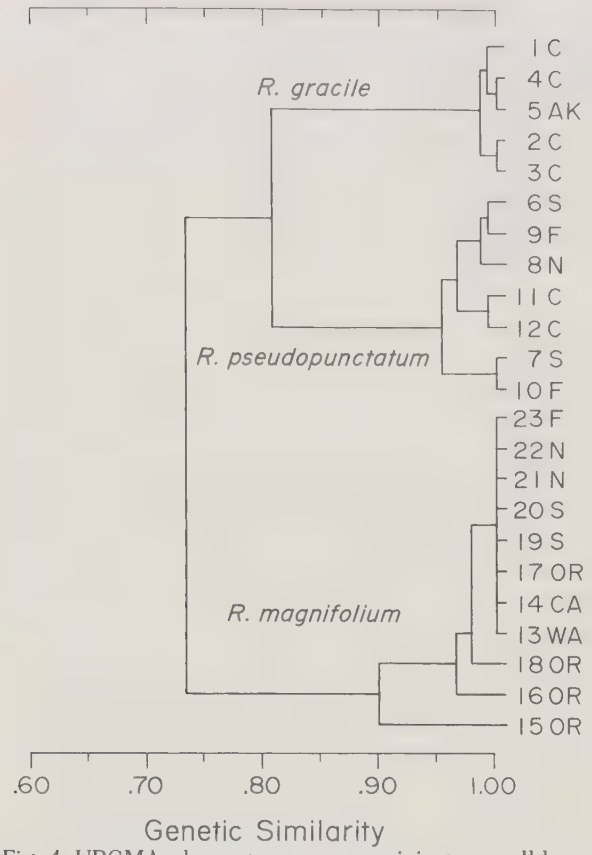

Fig. 4. UPGMA phenogram summarizing overall levels of genetic similarity among 23 populations of *Rhizomnium gracile* (1–5), *R. pseudopunctatum* (6–12), and *R. magnifolium* (13–23) based on Rogers' (1972) coefficient of genetic similarity using 11 putative gene loci. The cophenetic correlation coefficcient was 0.895. Abbreviations for states are: AK, Alaska; CA, California; OR, Oregon; WA, Washington. Abbreviations for countries are: C, Canada; F, Finland; N, Norway; S, Sweden.

somes in *R. pseudopunctatum* was approximately double that of *R. gracile* and *R. magnifolium*, it was difficult to obtain preparations that clearly separated all 14 chromosomes. Details of these counts and photographs will be published elsewhere (Stoneburner *et al.*, unpublished).

Discussion

All of the enzymes we screened in *R. gracile* and *R. magnifolium*, except TPI, are consistent with Gottlieb's (1982) model for vascular plants, which postulates that most plants express a predictable number of loci consistent with the subcellular compartmentation of their isozymes. For many enzymes with well-defined biochemical roles and activity in both chloroplastic and cytosolic forms, two isozymes are commonly expressed (Wendel and Weeden 1989). In most cases, however, we were able to detect and score consistently under our electrophoretic conditions only one of the two isozymes. It appears that the complex 7-banded phenotypes observed for TPI are the result of duplication of both the cytosolic and organellar genes for this enzyme. Single-gene duplications of both TPI isozymes also occur in *Plagiomnium* section *Rosulata* (Wyatt *et al.* 1992). Unfortunately, there is not sufficient genetic variation at these loci in *Rhizomnium* to confirm our interpretations. We have therefore taken a conservative approach and scored only the fastest and slowest putative loci (Tpi-1 and Tpi-4).

It is misleading to use standard population genetic statistics to compare levels of genetic variation when an allopolyploid like *R. pseudopunctatum* is involved. Much of the apparent variation is actually present in a fixed heterozygous state. It, therefore, does not segregate at individual gene loci, but rather can only be recombined across loci, manifesting itself as different multilocus genotypes. For this reason, our discussion of levels of genetic variation and its partitioning among populations will focus primarily on the haploid species, *R. gracile* and *R. magnifolium*.

Overall levels of genetic variation in these two species are remarkably low. Compared to other mosses studied using the same techniques, the mean percentage of loci polymorphic per population, mean number of alleles per locus, and mean expected heterozygosity are lower than for any other moss studied to date (Wyatt *et al.* 1989b; Stoneburner *et al.* 1991). The values actually are more similar to those reported for liverworts, which typically average nearly an order of magnitude less variation than mosses. The means for liverworts, however, are very heavily weighted by studies of boreal species, whose populations all occur north of the glacial boundary in Europe. Perhaps this offers at least a partial explanation for the extremely low levels of genet-

ic variation in *R. gracile* and *R. magnifolium*. All of the populations of these boreal species that we studied came from regions that were deglaciated only 5-8 x 10^3 years B. P. It is possible that the genetic effects of possible bottlenecks at that time have had profound influence on present-day levels of genetic variation. On the other hand, we found no evidence of reduced levels of genetic variation in the circumboreal *Plagiomnium ellipticum* (Wyatt 1992; Wyatt *et al.* 1992).

Because of its unusual hybrid genome, we were not surprised that *R. pseudopunctatum* shows remarkably little differentiation across its circumboreal range. Despite our having sampled populations from both Europe and North America, < 5% of the total variation existed as differences among populations. G_{ST}s were also very low for *R. gracile* (0.024), despite sampling of populations > 4,000 km apart. The somewhat higher G_{ST} for *R. magnifolium* (0.149) still would place it well below average for species of *Plagiomnium* in terms of population differentiation (Wyatt 1992). Its value is closest to that for the European endemic *P. elatum* (0.141). Recall, however, that populations of the circumboreal *R. magnifolium* were sampled from both northern Europe and western North America. Surprisingly, the strongest differentiation exists not between intercontinentally disjunct populations, but rather between populations in western North America. Again, it is possible that this curious pattern of low total genetic diversity and low differentiation among populations (except in unglaciated regions) is related to very recent range expansion of a species that underwent bottlenecks in population size in the recent past.

These species of *Rhizomnium* are remarkable in their very low degree of genetic divergence between populations, including intercontinentally disjunct populations. For *R. magnifolium* and *R. pseudopunctatum*, Nei's (1972) genetic identities between European and North American populations are all > 0.92. In contrast, Parks and Wendel (1990) reported a mean genetic identity of 0.43, ranging to a low of 0.32, between North American populations of *Liriodendron tulipifera* and East Asian populations of the vicariant species *L. chinense*. Similarly, Hoey and Parks (1991) reported genetic identities in the range 0.26–0.51 between intercontinentally disjunct species of *Liquidambar*. Crawford *et al.* (1992) have recently reviewed the literature on plant species disjunctions and concluded that intercontinentally disjunct species of flowering plants are typically very strongly differentiated electrophoretically. Only in unusual cases of recent long-distance dispersal are the disjunct taxa electrophoretically similar. This is so, despite the fact that the taxa may be morphologically nearly identical.

As discussed by Wyatt *et al.* (1992), there are at least two possible explanations for the discrepancy between mosses and flowering plants in terms of the degree of genetic divergence of widely isolated populations. First, it is possible that *R. magnifolium* and *R. pseudopunctatum* have only very recently (in the post-Pleistocene) expanded to occupy their present, circumboreal ranges. Although, as in the case of *Plagiomnium*, neither of these species seems especially well-suited to long-distance dispersal, many bryologists believe that spores and fragments of mosses can travel long distances (e.g., Zanten and Pócs 1981). Alternatively, the rate of accumulation of mutations may be much slower in mosses than in other land plants. We do not, however, have any reason to expect such a difference (Wyatt *et al.* 1992).

Perhaps the most important result from our isozyme studies of *Rhizomnium* is confirmation of the allopolyploid nature of *R. pseudopunctatum*. Equally clear is the indication that the progenitor stocks that hybridized to launch this new species were genetically very similar to present-day populations of *R. gracile* and *R. magnifolium*. Elsewhere we will report data on other taxa of *Rhizomnium* section *Rhizomnium* that clearly eliminate them as the possible contributors of the alleles that comprise *R. pseudopunctatum*. After initial screening of

eight taxa, it became apparent that only *R. gracile* and *R. magnifolium* could have contributed the alleles present in fixed heterozygous condition in *R. pseudopunctatum*, so we concentrated our studies on those three taxa.

Five of the 16 loci surveyed provided unequivocal evidence that *R. pseudopunctatum* is an allopolyploid derived from interspecific hybridization between *R. gracile* and *R. magnifolium* (or, technically, the recent ancestors of extant populations of those two species). Three of these involve fixed heterozygosity between alleles that are monomorphic in all populations of the progenitors. Two others involve fixed heterozygosity between the most common alleles of the progenitors. At the 11 other loci, the species are either fixed for the same allele or express the same most common allele.

Two of the three orphan alleles detected in populations of *R. pseudopunctatum* were highly limited in both frequency and geographical occurrence: Got-1c occurred in only one heterozygous plant from Kongsvold, Norway, and Pgm-1c was detected in about a dozen heterozygous plants in each of two populations from northern Ontario, Canada. These probably represent recent mutations, like the rare G3p-2b allele of *Plagiomniun medium* (Wyatt *et al.* 1992). On the other hand, it is possible that the Pgm-1c allele, like the third orphan allele (Mdh-1a), once occurred in populations of *R. gracile* or *R. magnifolium* and was subsequently lost. Mdh-1a occurs at high enough frequencies in most populations of *R. pseudopunctatum* as to make it less likely that it represents a new mutation. We predict that with more intensive sampling, especially of *R. gracile*, we may discover some populations that harbor these alleles (cf. Aco-1d of Wyatt *et al.* 1992).

There is one additional piece of information that is problematical in our interpretation of *R. pseudopunctatum*. Populations of *R. gracile* are fixed for the Aco-1b allele, whereas only Aco-1a has been detected in *R. magnifolium*. Thus, all plants of *R. pseudopunctatum* should be fixed heterozygotes at this locus. More than half do show this pattern, but a substantial number appear to express only Aco-1b. An explanation for this pattern is that the genetic contribution of *R. magnifolium* has been silenced in these plants, a phenomenon that seems to be common in polyploid pteridophytes (Haufler and Soltis 1986: Haufler 1987, 1989; Gastony 1991) but uncommon in mosses (Wyatt *et al.* 1992). Alternatively, it is possible that the simplified Aco-1 genotypes we observed were, in fact, homozygotes. This view would imply that the Aco-1a allele occurs (or occurred at the time of origin) in populations of *R. magnifolium*. It would also provide evidence, which is lacking at the present time, for multiple origins of *R. pseudopunctatum*. Unlike the situation in *Plagiomnium medium*, where numerous loci provided unequivocal evidence of multiple origins, low genetic polymorphism in species of *Rhizomnium* precludes any certainty on the issue of multiple origins of *R. pseudopunctatum*.

Lack of sufficient genetic polymorphism also limits our ability to pinpoint the time(s) and place(s) of origin of allopolyploid *R. pseudopunctatum*. Given the lack of definitive evidence for silencing of duplicated genes, the low number of orphan alleles (despite limited population sampling from within very wide ranges), and the very low degree of divergence among populations, it appears that *R. pseudopunctatum* is a recent derivative of *R. gracile* x *R. magnifolium*. The likely locus of origin is North America, where the two species presently overlap to a limited extent in Canada (Koponen 1973). Presumably, the species may have occurred sympatrically to the south of their present ranges during the Pleistocene. They may also have overlapped in unglaciated regions of Alaska and China, as Koponen and Afonina (1990) have recently reported *R. gracile* from Asia. Similar scenarios have been proposed to explain hybrid speciation in *Plagiomnium* (Wyatt *et al.* 1988, 1992) and *Dryopteris* (Werth 1991).

In conclusion, our evidence strongly sup-

ports Koponen's (1973) view that *R. pseudopunctatum* is closely related to *R. gracile* and *R. magnifolium*. It does not support Lowry's (1948) original hypothesis that *R. punctatum* was involved in the origin of *R. pseudopunctatum*. The discovery of this second example of allopolyploid speciation in the Mniaceae suggests that interspecific hybridization in mosses is more common than had originally been supposed.

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APPENDIX

The table below gives sample sizes (N) and allele frequencies for 16 loci in five populations (1–5) of *Rhizomnium gracile*, seven populations (6-12) of *R. pseudopunctatum*, and eleven populations (13–23) of *R. magnifolium*. Abbreviations for enzyme loci are given in the text. Population codes are given in Table 3. Asterisks denote missing data. Note that allele frequencies for the allopolyploid *R. pseudopunctatum* do not represent segregating polymorphisms, but rather alleles present in fixed heterozygous genotypes.

		Population											
Locus		1	2	3	4	5	6	7	8	9	10	11	12
Aco-1	(N)	30	30	30	*	14	96	106	90	94	96	24	68
	a	—	—	—		—	0.50	0.29	0.26	0.21	0.31	0.13	0.21
	b	1.00	1.00	1.00		1.00	0.50	0.71	0.74	0.79	0.69	0.87	0.79
Ald-1	(N)	*	10	*	*	14	*	*	16	20	*	*	*
	a		1.00			1.00			1.00	1.00			
Fle-1	(N)	48	45	48	44	14	*	*	90	94	96	96	68
	a	0.78	1.00	1.00	1.00	1.00			1.00	1.00	1.00	1.00	1.00
	b	0.02	—	—	—	—			—	—	—	—	—
G3p-1	(N)	*	10	*	*	14	*	*	16	20	*	*	*
	a		1.00			1.00			1.00	1.00			
Got-1	(N)	48	45	48	44	6	96	110	90	94	96	96	68
	a	—	—	—	—	—	—	—	—	—	—	—	—
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.96	1.00	1.00	1.00	1.00
	c	—	—	—	—	—	—	—	0.04	—	—	—	—
Hex-1	(N)	48	45	48	44	14	96	110	90	94	96	96	68
	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdh-1	(N)	48	45	48	44	8	96	110	90	94	96	96	68
	a	—	—	—	—	—	—	0.50	0.08	0.04	0.50	0.24	0.22
	b	1.00	1.00	1.00	1.00	1.00	1.00	0.50	0.92	0.96	0.50	0.76	0.78
Me-1	(N)	48	45	48	44	14	96	110	90	94	96	96	68
	b	—	—	—	—	—	0.50	0.50	0.50	0.50	0.50	0.50	0.50
	c	1.00	1.00	1.00	1.00	1.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mpi-1	(N)	48	45	48	44	14	*	*	90	94	96	96	68
	c	1.00	1.00	1.00	1.00	1.00			1.00	1.00	1.00	1.00	1.00
Per-1	(N)	48	45	48	44	*	96	110	90	94	96	96	32
	a	—	—	—	—		0.50	0.50	0.50	0.50	0.50	0.50	0.50
	c	1.00	1.00	1.00	1.00		0.50	0.50	0.50	0.50	0.50	0.50	0.50
Pgd-1	(N)	*	10	*	*	14	*	*	*	20	*	*	*
	a		1.00			1.00				1.00			
Pgi-2	(N)	48	47	48	44	14	96	110	90	94	96	96	68
	b	0.02	0.06	0.06	—	—	—	—	—	—	—	—	—
	c	0.98	0.94	0.94	1.00	1.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50
	e						0.50	0.50	0.50	0.50	0.50	0.50	0.50
	f	—	—	—	—	—	—	—	—	—	—	—	—

(continued)

Appendix *(continued)*

Pgm-1	(N)	48	45	48	44	14	96	110	90	94	96	96	68
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.93	0.91
	c	—	—	—	—	—	—	—	—	—	—	0.07	0.09
Pgm-2	(N)	48	45	48	44	14	96	110	90	94	96	96	68
	a	—	—	—	—	—	0.50	0.50	0.50	0.50	0.50	0.50	0.50
	b	1.00	1.00	1.00	1.00	1.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Tpi-1	(N)	48	45	48	44	14	96	110	90	94	96	96	68
	c	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	d	—	—	—	—	—	—	—	—	—	—	—	—
Tpi-4	(N)	48	45	48	44	8	96	110	90	94	96	96	68
	d	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

		Population										
Locus		13	14	15	16	17	18	19	20	21	22	23
Aco-1	(N)	64	84	72	96	23	74	23	92	46	17	47
	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	—	—	—	—	—	—	—	—	—	—	—
Ald-1	(N)	*	*	*	10	*	*	*	*	8	*	*
	a				1.00					1.00		
Fle-1	(N)	*	*	*	10	*	*	*	*	54	17	47
	a				1.00					1.00	1.00	1.00
	b									—	—	—
G3p-1	(N)	*	*	*	10	*	*	*	*	8	*	*
	a				1.00					1.00		
Got-1	(N)	64	84	72	93	23	71	45	92	46	17	47
	a	—	—	0.90	0.28	—	—	—	—	—	—	—
	b	1.00	1.00	0.10	0.72	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	c	—	—	—	—	—	—	—	—	—	—	—
Hex-1	(N)	64	84	72	96	24	74	45	92	46	17	47
	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdh-1	(N)	64	82	72	96	24	74	45	92	46	17	47
	a	—	—	—	—	—	—	—	—	—	—	—
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Me-1	(N)	64	84	72	96	24	74	45	92	46	17	47
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	c	—	—	—	—	—	—	—	—	—	—	—
Mpi-1	(N)	*	*	*	*	*	*	*	*	46	17	47
	c									1.00	1.00	1.00
Per-1	(N)	64	84	72	96	24	53	45	92	46	17	47
	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	c	—	—	—	—	—	—	—	—	—	—	—
Pgd-1	(N)	*	*	*	10	*	*	*	*	*	*	*
	a				1.00							
Pgi-2	(N)	64	36	36	48	12	41	23	45	46	17	47
	b	—	—	—	—	—	—	—	—	—	—	—
	c	—	—	—	—	—	—	—	—	—	—	—
	e	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	f	—	0.03	—	—	—	—	—	—	—	—	—
Pgm-1	(N)	64	84	71	94	23	74	45	88	46	17	47
	b	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	c	—	—	—	—	—	—	—	—	—	—	—
Pgm-2	(N)	32	36	36	48	12	41	23	45	46	17	47
	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	—	—	—	—	—	—	—	—	—	—	—
Tpi-1	(N)	64	84	72	96	24	68	45	92	46	17	47
	c	1.00	1.00	1.00	1.00	1.00	0.79	1.00	1.00	1.00	1.00	1.00
	d	—	—	—	—	—	0.21	—	—	—	—	—
Tpi-4	(N)	64	84	72	96	24	68	45	92	46	17	47
	d	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Gravitropism in protonemata of the moss *Ceratodon* [1]

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SACK, F. D. (Department of Plant Biology, Ohio State University, Columbus, OH 43210). Gravitropism in the protonemata of the moss *Ceratodon*. Mem. Torrey Bot. Club 25(1): 36–44. 1993. – Protonemata of *Ceratodon* grow up in the dark when cultured on nutrient supplemented media. Both gravitropic sensing and differential growth (curvature) occur in or close to the tip of the apical cell, and tip growth is tightly coupled to tip orientation. When protonemata are reoriented 90°, the tip grows down slightly (wrong-way curvature) before then curving up. In horizontal protonemata, there is extensive sedimentation of amyloplasts in a specific zone located behind the apical dome. This sedimentation could function in gravitropic sensing since it precedes upward curvature. This hypothesis is also supported by data from centrifugation experiments. However, little or no sedimentation occurs before wrong-way curvature; thus, if sensing for this earlier phase of gravitropism relies upon amyloplast mass, then extensive sedimentation is not necessary. Comparison of upright and inverted cells shows that some amyloplast sedimentation also occurs in vertical cells, especially between the sedimentation zone and the nucleus. However, amyloplasts do not sediment completely through vertical cells except when protonemata are treated with microtubule inhibitors. Thus, microtubules influence the direction and extent of amyloplast sedimentation. In upward curving cells, microtubules become enriched in the lower flank of the Golgi zone behind the apical dome. We hypothesize that amyloplast sedimentation induces an enrichment in microtubules that results in upward growth.

Key Words: *Ceratodon*, gravitropism, moss, protonemata, tip growth

Since plants are not motile, their survival depends in large part on orienting their growth and form with respect to gravity. Gravitropism is clearly adaptive in orienting organs with respect to minerals, water and light. This orientation is adaptive for mosses as well as for higher plants. For example, the upward growth (negative gravitropism) of the moss sporophyte aids in spore dispersal. As might be expected, research on gravitropism in mosses has been relatively sparse. Gametophores and sporophytes have received some study (Petschow, 1933; Banbury, 1962), but until the last few years almost nothing was known about gravitropism in moss protonemata. This paper reviews current knowledge of protonematal gravitropism, especially for *Ceratodon purpureus*.

Protonemata of mosses such as *Physcomitrella* and *Ceratodon*, constitute one of the few gravitropic single-celled systems known in the plant kingdom. The other system that has been described is the rhizoid of the alga *Chara* (Sievers and Volkmann, 1979). Moss protonemata are negatively gravitropic (grow up) whereas the *Chara* rhizoid grows down. All these single-celled systems are tip-growing cells where cell extension is confined to the apical dome of the tip cell. Of course, all such apical cells are attached to other cells at their base, but it is the growing tip itself whose orientation influences its own growth. Thus, the term "single-cell" is used in the sense that sensing and the gravitropic response occur in the same cell.

When *Ceratodon* protonemal fragments are transferred to sucrose-supplemented agar and the surface of the agar is placed in a vertical orientation, hundreds of straight and upright protonemata are formed in a few days. When protonemata are reoriented to the horizontal, upward curvature soon follows (Walker and Sack 1990). The goal of our research with *Ceratodon* gravitropism is to understand the cellular events that pro-

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[2] Work by, and discussions with, Donggium Kim, Jochen Schwuchow, Loni Walker, and Jeff Young are gratefully acknowledged.

duce this gravitropism. Work to date has shown that this phenomenon is complex and fascinating, and has revealed some clues as to which cell components function in different phases of gravitropism.

Gravitropism is essentially oriented elongation. It seems to exist in three phases (Sack 1991), i.e. *sensing*, the action of gravity upon some mass, *transmission* of a gradient carrying information from the sensing phase to the target region, and the *response* which is the modulated growth of the target region (e.g. differential growth producing curvature). Much might be gained from studying gravitropism in tip-growing cells, since sensing and differential growth occur close together, whereas in organs these sites are separated by several to many cells. Thus, in single-celled systems, gravity-induced cellular asymmetries are compressed into a few micrometers, and this enhances the identification of the gradients essential for gravitropism. This identification would help us understand the cell biology of regular tip-growth as well as gravitropism.

Protonemata Grow Up in the Dark

The negative gravitropism of *Ceratodon* protonemata was first reported by Hartmann (1984). When straight protonemata are reoriented to the horizontal, new upward curvature starts after 30–60 minutes and the majority of protonemata reach 90° by 16 hours (Fig. 1; Walker and Sack 1990). This gravitropism is inhibited by all wavelengths of visible light tested (Young and Sack 1992). Thus, to observe gravitropism, protonemata were either grown in the dark and then fixed *in situ*, or individual, living protonemata were observed using infrared videomicroscopy and a horizontally-mounted microscope. Near infrared light has no effect on gravitropism (Young and Sack 1992). This videomicroscopy method is a modification of that used by Knight and Cove (1991) to study the negative gravitropism of protonemata of the moss *Physcomitrella*.

Plastid Zonation and Gravity

Dark grown *Ceratodon* protonemata exhibit a complex plastid zonation and contain plastids that react differently to gravity depending upon their location along the length of the cell (Walker and Sack 1990). There are 3 plastid zones starting from the very tip of the apical cell and proceeding towards the base of the cell (Fig. 1, one hour): (1) a cluster of non-sedimenting amyloplasts in the apical dome, (2) a relatively plastid-free zone, and then (3) a zone of distinct amyloplast sedimentation. Quantitation of plastid position indicates that sedimentation occurs primarily in this third zone and that sedimentation is negligible or absent in other zones in horizontal protonemata (Walker and Sack 1990).

Amyloplast sedimentation is not obvious in vertical protonemata since amyloplasts do not fall completely through the length of the cell. In contrast, amyloplasts in zone 3 do sediment completely to the lower (side) wall in horizontal protonemata. This observation led us to propose originally that the sedimentation zone appears more specialized for lateral than for axial sedimentation (Walker and Sack 1990). However, recent work, discussed below, shows that plastid sedimentation can also occur in an axial direction.

The plastid zonation found in dark-grown apical cells of *Ceratodon* has not been described for other mosses. Dark-grown protonemata in other genera lack a distinct group of plastids in the apical dome (Jensen and Jensen 1984; Schmiedel and Schnepf 1980; Tewinkel and Volkmann 1987; DeMaggio and Stetler 1977; Doonan *et al.* 1988).

Amyloplasts May Function in Gravity Sensing

The presence of amyloplast sedimentation in yet another gravitropic system strongly reinforces the correlation between these two events throughout the plant kingdom (Sack 1991). In addition to gravitropic protonemata, in mosses amyloplast sedimentation also occurs in gravitropic game-
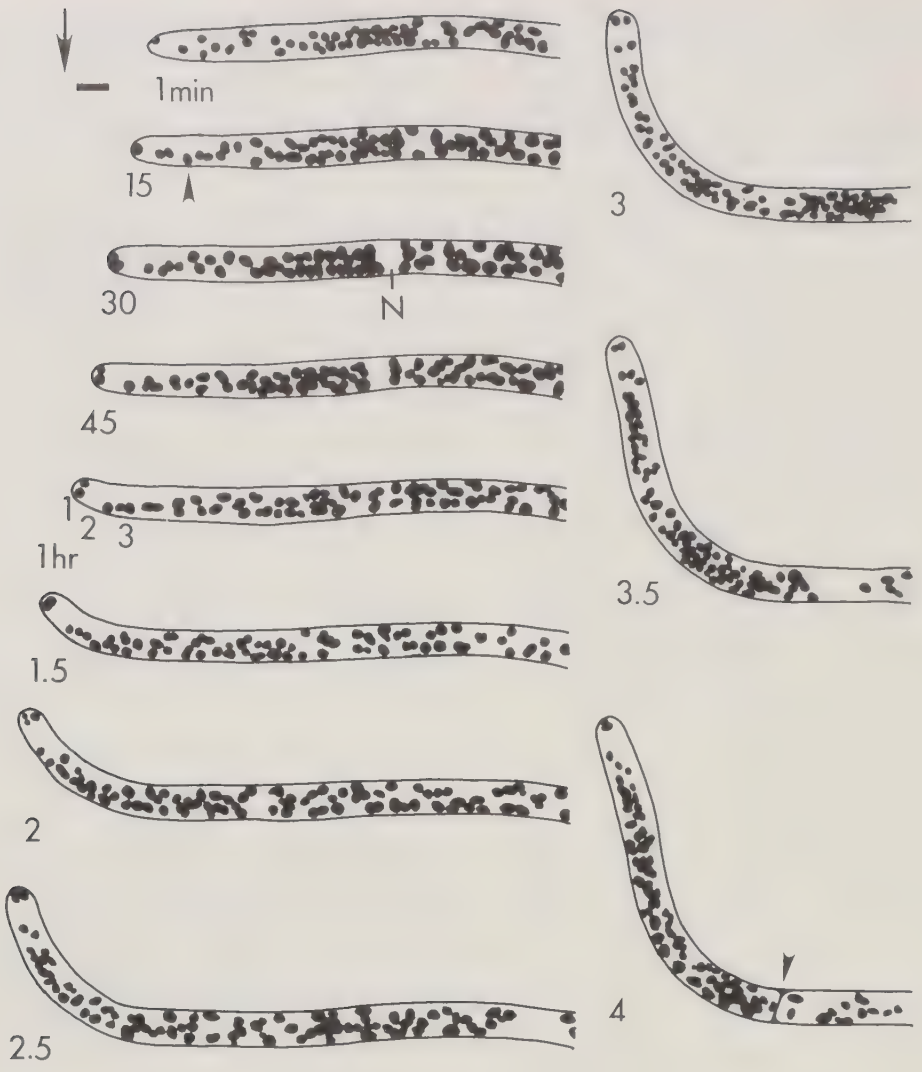
Fig. 1. Time course of upward curvature (negative gravitropism) of a single protonema of *Ceratodon* during the 4 hr following horizontal placement. Tracing from Fig. 2 in Young and Sack (1992) depicting a series photographed from the video monitor. The gravity vector (arrow, 1 min) is toward the bottom. Times (in min or hr) after horizontal placement are indicated below each tracing. Each tracing is aligned so that protonemal position (old growth) is fixed and new tip growth (extension) through time is visible. The three plastid zones found in the apical part of the cell are numbered (at 1 hr). Note that some amyloplast sedimentation (arrowhead at 15 min) preceded the start of upward curvature which is noticeable at 45 min. Amyloplast sedimentation continued in zone three throughout most of the course of upward curvature and was not obvious in other zones. The distance between the apex and the first sedimented amyloplast stayed relatively constant as the tip curved up (compare 45 min with 2 hr). Division of the tip cell resulted in a temporary loss of curvature, e.g., compare 3.5 and 4 hr with 3 hr (new cell wall shown by arrowhead at 4 hr). Magnification and time resolution insufficient to show wrong-way curvature.

N = nucleus. Bar = 20 μm

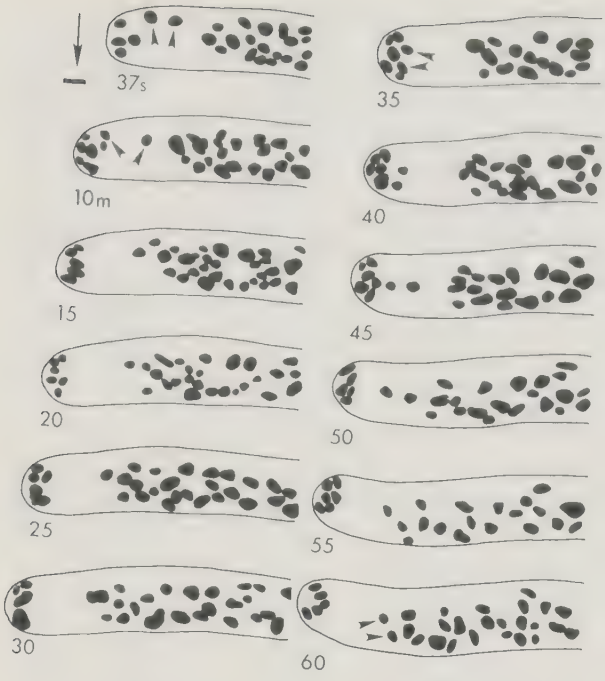

Fig. 2. Rapid wrong-way curvature followed by the onset of upward curvature during the 1 hr following horizontal placement. Tracing from Fig. 5 in Young and Sack (1992). The first tracing of the series is 37 sec after rotation to the horizontal. Downward flattening of the upper flank can be seen at 10 min, especially compared to the lower wall. This was followed by a slight downward curvature of the whole tip (20–25 min) and then by upward curvature. Upward curvature is well defined by 60 min and continued after that time (not shown). Often plastids moved basipetally from the apical dome and from zone two to the sedimentation zone (right arrowhead in 37 sec and 10 min; arrowheads in 35 and 60 min), but sometimes movement was acropetal (left arrowheads in 37 sec and 10 min). Some plastid sedimentation occurred before the start of upward curvature, and the sedimentation increased with time.

Bar = 10 μm

tophores and sporophytes (Petschow 1933; Banbury 1962). As is well known, it is thought that amyloplasts that sediment provide the mass that functions in gravitropic sensing in organs and that these amyloplasts are said to be "statoliths". Thus, these correlations have been interpreted to represent a causal relationship.

But it has recently been found that some mutants of higher plants are starch-deficient yet gravitropic (Kiss, Hertel, and Sack, 1989; Kiss and Sack 1989). This important finding establishes that amyloplasts and starch are not necessary for gravitropic sensing in some higher plant organs. However, it is likely that amyloplasts contribute to sensing in wild-type higher plants since gravitropic sensitivity was depressed in the starch-deficient mutants. Thus, these data support the classical hypothesis that amyloplasts, starch and sedimentation function in sensing, although this hypothesis also clearly needs revision (Sack 1991).

In *Ceratodon* protonemata, additional correlations support the hypothesis that amyloplasts that sediment function as statoliths for upward curvature. The kinetics of amyloplast sedimentation are consistent with the idea that these plastids are statoliths. In populations of protonemata, amyloplast sedimentation in zone 3 started about 15 minutes after reorientation and this preceded the start of upward curvature (30–60 minutes; Walker and Sack 1990). Study of individual protonemata by time-lapse video always showed sedimentation before upward curvature (Figs. 1, 2; Young and Sack 1992). Furthermore, sedimentation took place within the threshold stimulation time (presentation time) of 12–17 minutes. Sedimentation continued throughout the extended period of upward curvature.

A second set of supporting data comes from centrifugation studies. Following centrifugation of the tip amyloplasts out of the tip and to the basal wall, tip growth resumed at rates comparable to control (uncentrifuged) protonemata (20–40 $\mu m \cdot h^{-1}$), but gravitropism was absent until amyloplasts migrated back close to the tip and sedimented (Walker and Sack 1991). Before migration back to the tip, many amyloplasts sedimented to the lower wall in the basal part of these horizontal cells, but this event was not correlated with upward curvature. Thus, it appears that plastid sedimentation must occur in a specific region for upward curvature to occur.

Collectively, these results suggest that those amyloplasts which sediment within these horizontal cells function as statoliths

in upward curvature. This work is the first modern description of a cell that both senses and responds to gravity which appears to employ amyloplasts in gravitropic sensing.

Wrong-Way Precedes Upward Curvature

Careful examination of the early events following reorientation to the horizontal of living *Ceratodon* protonemata shows that before the protonemata start to curve upward, virtually all protonemata first curve slightly downward (Fig. 2; Young and Sack 1992). Thus, the first visible response to reorientation was a flattening of the upper flank of the apical dome in 2–20 minutes; subsequently, this flattening usually developed into a downward ("wrong-way") curvature. Thus, the gravitropic response occurs in at least two phases.

Wrong-way curvature is common in gravitropic organs of higher plants but this is the first report for a single-celled system (Young and Sack 1992). More significantly, the rapidity and consistency of the early response shows that tip growth in *Ceratodon* is very tightly coupled to orientation. Wrong-way curvature in *Ceratodon* is one of the fastest growth responses to gravity known for the plant kingdom.

Also, wrong-way curvature occurs before there is any dramatic amyloplast sedimentation anywhere in the protonema. This appears to be the first report for any plant of a wild-type growth response to gravity that occurs *without* prior and significant amyloplast sedimentation (Sack 1991). Centrifugation experiments provide the hint that the plastids in the dome may be correlated with sensing for wrong-way curvature, since wrong-way curvature only took place if plastids were present in the apical dome (Walker and Sack 1991). Thus, plastid mass might function in gravity sensing in wrong-way curvature even without sedimentation, although sedimentation still appears to be required for the later phase of upward curvature.

Dynamism of Plastid Zonation

Time-lapse observation of living protonemata using infrared videomicroscopy reveals that plastid zonation in *Ceratodon* is highly dynamic. In real time, neither cytoplasmic streaming nor plastid movement could be detected. However, in time-lapse, almost all plastids were seen to move with different plastids moving towards the tip and others moving towards the base (Young and Sack 1992). Despite this movement, plastid zonation was maintained. For example, the position of the sedimented amyloplasts stayed a constant distance from the tip during growth and during curvature when these plastids moved up the lowermost wall. Plastid zonation was disrupted during mitosis, and this correlated with a transient reversal of upward gravitropic curvature (Fig. 1). Many of these observations could be explained if the cytoskeleton interacted with the plastids (see below).

Plastid Sedimentation in Vertical Cells

In upright, vertical protonemata, amyloplast sedimentation is not apparent since amyloplasts are distributed throughout the length of the cell. In other words, if amyloplast sedimentation occurs in upright cells, it is hard to see since the amyloplasts do not fall completely through the length of the cells. However, if upright cells are compared with inverted cells, it becomes clear that amyloplast sedimentation occurs in both orientations (Fig. 3 a–c). In both cases, sedimentation is incomplete. In upright cells there are more amyloplasts near the nucleus than in the tip. But amyloplasts in the apical dome do not fall out of the dome, and, in the sedimentation zone, only some amyloplasts fall towards the nucleus. Most of these amyloplasts do not sediment past the nucleus to the lowermost wall. Conversely, in inverted cells, some amyloplasts sediment as far as the plastid-free zone, but none appear to fall into the apical dome. Thus, plastid sedimentation occurs in all directions, but sedimentation takes place in only some zones, and the extent

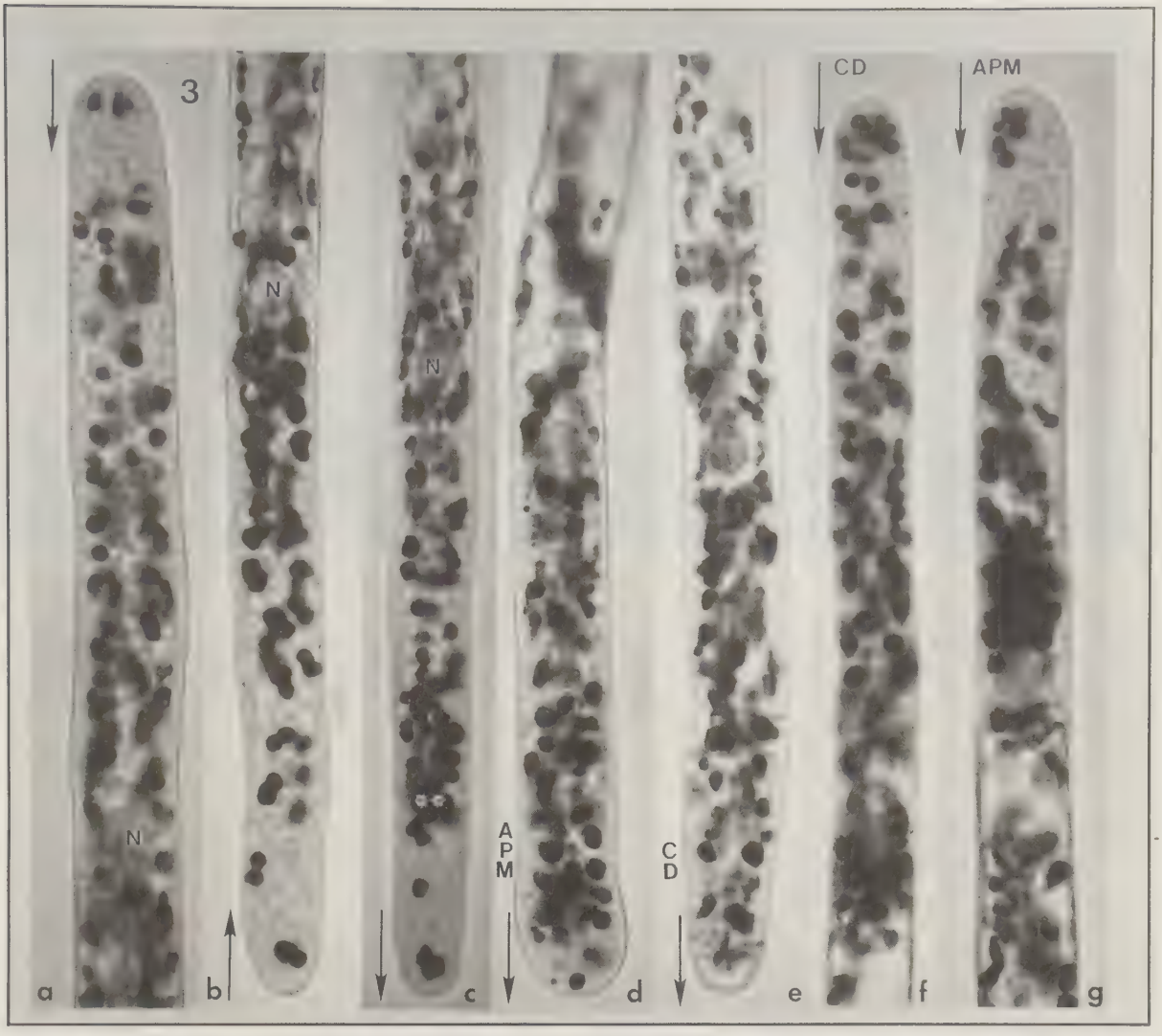

Fig. 3. The effects of inversion and of cytoskeletal inhibitors on amyloplast sedimentation in *Ceratodon* protonemata. Protonemata were either maintained upright (a, b, f, and g) or were inverted (c – e). The arrow indicates the gravity vector. In Fig. b, the micrograph of an upright cell was rotated 180° to facilitate comparison with the inverted cell shown in c. Protonemata were fixed in position (in the dark) with glutaraldehyde and then starch was stained with iodine potassium iodide. In c, note the increased sedimentation of the amyloplasts (*) towards the tip upon inversion. Conversely, upright protonemata (a & b) have more amyloplasts close to the nucleus (N) compared to inverted cells.

The addition of the microtubule depolymerizing drug amiprophos-methyl (APM) enhanced plastid sedimentation along the length of cells that were inverted (d) or upright (g). The microfilament depolymerizing drug, cytochalasin D (CD) disrupted plastid zonation (note the absence of a plastid-free zone), but did not enhance plastid sedimentation in either inverted (e) or upright (f) cells. Micrographs by Jochen Schwuchow. All protonemata are shown at the same magnification. Bar (a, upper right) = 10 µm.

and frequency of sedimentation is highly regulated. (Schwuchow and Sack, in press).

That the cytoskeleton is involved in this regulation is demonstrated by the application of chemicals which depolymerize microtubules and microfilaments. When the microtubule inhibitor APM (amiprophos-methyl; Schwuchow et al. 1990) is applied to upright or inverted cells, amyloplast sedimentation is enhanced (Fig. 3 b–d, g); at higher APM concentrations, amyloplasts sediment completely to the lowermost wall (data not shown). The microfilament inhibitor cytochalasin D did not enhance sedimentation compared to untreated cells, but it did eliminate the plastid-free zone behind the apical dome (Fig. 3 e–f). These data suggest that microfilaments help main-

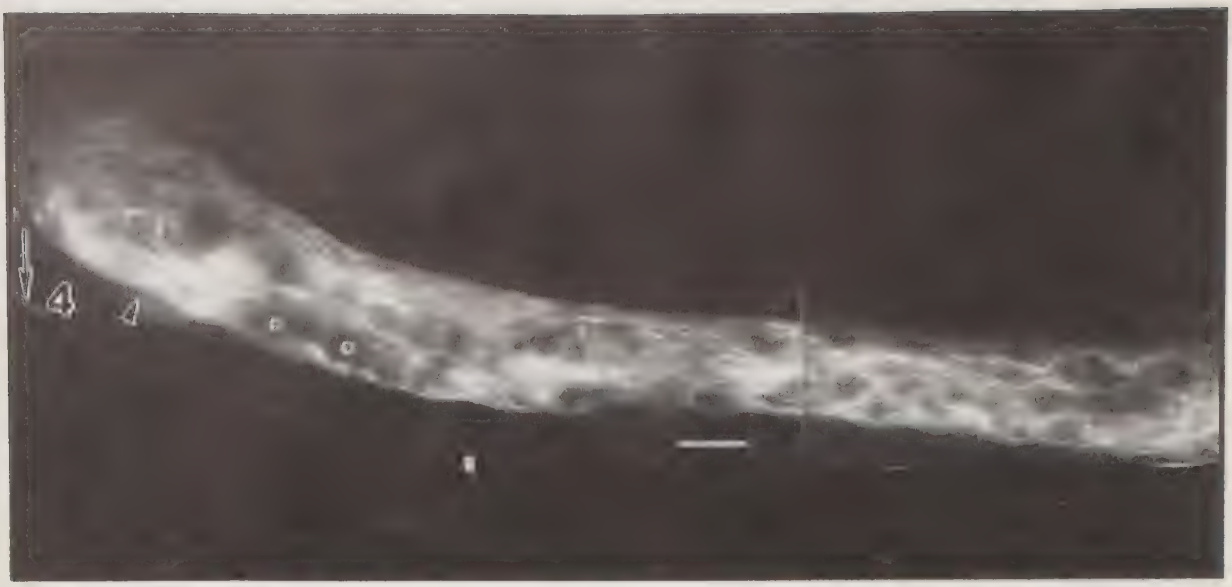

Fig. 4. Protonema of *Ceratodon* stained for microtubules by immunofluorescence. The tip of the protonema is towards the left, and the gravity vector is indicated by the arrow at the left. The protonema was horizontal for several hours as is evidenced by the upward curvature shown in the left half of the cell in the figure and by the sedimented amyloplasts distinguishable by the absence of tubulin immunofluorescence (*). There is a rich network of microtubules including in the apical dome. Note the brighter fluorescence from the enrichment in microtubules located in the lower flank of the plastid-free zone (arrowhead). Micrograph by Jochen Schwuchow. Bar = 10 m.

tain plastid zonation, and that microtubules regulate the extent of plastid sedimentation along the length of the cell.

In addition to establishing a central role for microtubules in controlling plastid sedimentation, these data also demonstrate the importance of the cytoskeleton in maintaining organelle position and cell organization against the force of gravity. Although it has been proposed that the cytoskeleton evolved, in part, to prevent the stratification of cell components with respect to gravity, there are few direct proofs of this role in the literature.

Microtubule Enrichment

Most *Ceratodon* protonemata that were horizontal for longer than 30 minutes appeared to have an enrichment in microtubules (detected with immunofluorescence) in the lower compared to the upper flank of the tip (Fig. 4; Schwuchow *et al.* 1990). This enrichment was located in the plastid-free zone and occurred both before and during upward curvature. Vertical protonemata lacked this enrichment i.e. they had a symmetrical distribution of microtubules across the tip.

It is not established that this microtubule enrichment functions in gravitropism, but it is interesting to speculate as to what role microtubules might play in gravitropism. It is possible that the increased concentration of microtubules somehow promotes the growth of the lower flank thereby producing upward curvature. Even though actual cell wall extension probably occurs closer to the tip than where the microtubule enrichment is located, microtubules could still promote growth by increasing the flow of exocytic Golgi vesicles on the lower side. Microtubules could differentially position this exocytosis either by providing tracks for wall-building vesicles, or by repositioning the Golgi stacks themselves. Electron microscopy reveals that the zone where the microtubules become enriched (plastid-free zone) is also a Golgi zone (Loni Walker, unpublished). Alternatively, microtubules might position and anchor ion channels in the plasma membrane; this could influence the site of secretion by affecting the concentration of calcium which could regulate vesicle fusion with the plasma membrane.

How might the microtubule enrichment come about? It is unlikely that microtubules themselves have sufficient comparative density to sediment in these cells. Perhaps amyloplast sedimentation physically brings down microtubules or enhances microtubule nucleation and polymerization in the lower flank.

A combined hypothesis for microtubules and amyloplast sedimentation could do much to explain how all three phases of upward curvature occur in these protonemata. Thus, sensing (the displacement of amyloplast mass by gravity) would be coupled to the response (differential growth) via a microtubule enrichment.

This hypothesis might also explain why protonemal gravitropism is temporarily reversed during mitosis (Fig. 1; Knight and Cove 1991; Young and Sack 1992). The mobilization of microtubules for the mitotic spindle might deplete the pool of tubulin available for the microtubule enrichment in the tip. The resulting loss of a microtubule enrichment might explain why upward curvature stops, but would not explain why curvature is actually reversed.

Gravitropism in other Moss Protonemata

Protonemata of *Physcomitrella* are also gravitropic, although they curve more slowly than *Ceratodon* (Jenkins *et al.* 1986; Knight and Cove 1991). *Funaria* protonemata are gravitropic as well (Sack, Tewinkel, and Volkmann, unpublished). Amyloplasts sediment in protonemata of both *Physcomitrella* and *Funaria* at locations comparable to that in *Ceratodon* (Tewinkel and Volkmann 1987; Kim and Sack, unpublished). Thus, gravitropism and amyloplast sedimentation may be widespread in dark-grown moss protonemata.

Moss protonemata offer much potential for understanding the cell biology of gravitropism. Hopefully this potential will be expanded as the tools of molecular biology are successfully extended to mosses. Already, much of the genetics of gravitropism in *Physcomitrella* protonemata have been elucidated (Jenkins *et al.* 1986). Of great interest to future research is a fascinating mutant of *Physcomitrella*, isolated by Knight *et al.* (1991), whose protonemata are gravitropic but which grow down instead of up.

Concluding Comments

Several generalizations emerge from this work with gravitropism in moss protonemata:

1) At least three moss genera have gravitropic protonemata. In dark-grown protonemata, tip growth is tightly coupled to orientation.

2) Amyloplast sediment in tip cells in all gravitropic moss protonemata. This correlation between sedimentation and gravitropism supports the hypothesis that amyloplast mass functions in gravitropic sensing.

3) In *Ceratodon*, centrifugation experiments suggest that amyloplast sedimentation is specifically important for upward gravitropic curvature.

4) *Ceratodon* protonemata curve slightly downward before curving upward and before significant amyloplast sedimentation takes place. Thus, if sensing for this earlier phase of gravitropism relies upon amyloplast mass, then extensive sedimentation is not necessary.

The evidence of a microtubule enrichment in curving protonemata suggests that microtubules might play a causal role in adjusting tip growth. And data with cytoskeletal inhibitors clearly indicate that microtubules are a primary agent in regulating the extent of plastid sedimentation. Thus, gravity may have acted as a selection pressure on the evolution of the cytoskeleton to both maintain cell organization and to function in protonemal gravitropism.

Finally, as we understand more about the cell and molecular biology of gravitropism in single-celled systems, we should learn

more about a fundamental unsolved problem in plant cell biology i.e. about how growth is polarized and maintained in a specific locus in a tip-growing cell.

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Diversity of cell division in simple land plants holds clues to evolution of the mitotic and cytokinetic apparatus in higher plants

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BROWN, R. C. AND B. E. LEMMON (Department of Biology, University of Southwestern Louisiana, Lafayette, LA 70504-2451). Mem. Torrey Bot. Club 25(1): 45–62. 1993. – The cytoskeletal structures that characterize the cell cycle in vegetative tissues of higher plants—cortical array of interphase, preprophase band of microtubules (PPB), spindle, phragmoplast—are highly evolved. Study of the lower land plants (bryophytes and lycopsids) has provided some clues to the divergent evolution of cytoskeletal structures associated with cell division in plants and animals. The diverse mechanisms of cell division surviving in lower land plants suggests a stepwise evolution from typical algal mitosis and cytokinesis. Hoop-like cortical microtubules, anastral spindles, and structures resembling PPBs and phragmoplasts are all present in certain extant algae, and are firmly established in the bryophytes. Within extant members of the bryophytes, we can trace steps in the evolution of the diffuse plant microtubule organizing center (MTOC) and introduction of the PPB into the cytokinetic apparatus. In bryophytes, the material associated with microtubule organization has come to be associated with membranes, e.g. endoplasmic reticulum in polar organizers, the plastid envelope in monoplastidic cells, or the nuclear envelope in polyplastidic cells. Some understanding of the existence of highly specialized ways in which plant cells control the cycling and organization of tubulin can be gained by studying nature's experiments in the lower land plants where diversity serves to emphasize commonalities.

Keywords: bryophytes, cytokinetic apparatus, lycopsids, microtubules, mitotic/meiotic apparatus, MTOC

The cytoskeleton in dividing cells of land plants is sequentially rearranged into distinct configurations during the cell cycle. The putative microtubule organizing centers (MTOCs) (Pickett-Heaps 1969) that control the cycling of tubulin in plants are diffuse and it is difficult to identify homologies with models of animal cell division where microtubule nucleation and organization is centered on centrosomes. Numerous proteins associated with the centrosome of animal cells have been identified and some progress has been made toward following their movement during the cell cycle (e.g. Kellogg *et al.* 1989; Kallajoki *et al.* 1992). Studies on the biochemistry of the plant MTOC are just beginning (Chevrier *et al.* 1992) and there are no comparable data on proteins associated with the anastral spindles of higher plants or with the unique cytokinetic apparatus.

The enormous amount of information on the cytoskeleton has been summarized in several recent books and review articles. Some aspects of the cytoskeleton in bryophytes have been dealt with in recent reviews of mitosis (Steer 1984), meiosis and sporogenesis (Brown and Lemmon 1988a) and protonemata (Doonan and Duckett 1988; Doonan 1991). The present paper emphasizes the contributions from studies of the lower plants (bryophytes and fern allies) to our knowledge of development, organization and function of the cytoskeleton in division. Comparative studies have shown that the lower land plants are a rich source of variation in the cytoskeletal structures associated with cell division. The bryophytes are the simplest extant land plants and as such, afford the opportunity to study evolution of the specialized mechanisms of cell division characteristic of higher plants. It is within this diverse assemblage that we can trace the evolution of the diffuse plant MTOC and the introduction of a predictive component, the preprophase band of microtubules (PPB), into the cytokinetic apparatus. The loss of centrioles and the appearance of the phragmoplast, a distinctive cytoskeletal structure associated with the deposition of new walls, can be traced to putative algal ancestors. Not any one bryophyte or alga can answer all of the questions and the record is fragmentary,

but taken together, clues provided by the rich diversity of mechanisms in cell division lead to generalizations that are useful in reconstructing major events that may have occurred.

Cell Cycle of Higher Plants.
The plant cell cycle is characterized by an orderly succession of microtubule arrays. In the cells of vegetative tissues dividing mitotically, four distinct systems of microtubules are generally recognized: the cortical array of interphase, preprophase band, division spindle and phragmoplast (Lloyd and Barlow 1982; Lloyd 1987; Staiger and Lloyd 1991). Each assembly is associated with important events such as cell shaping, establishment of division plane, chromosome movement and cell plate formation. Considering the fundamental importance of the cycling of tubulin in plant cells, the nucleation, organization and reorientation of plant microtubules is a central issue in plant cell biology. Of the four major arrays of microtubules in the plant cell cycle, only the mitotic spindle is clearly homologous to that in animal cells and even here significant differences can be seen in ontogeny and polar organization.

Cortical Array. The hoop-like cortical array of microtubules that characterizes walled plant cells in interphase is a specialized system associated with elongation of cells in a tissue (for review see Seagull 1989). The microtubules lie generally perpendicular to the axis of elongation and, in an elongating organ such as a root, the planes of cell division are principally parallel to the cortical microtubules. Reorganization of microtubules accompanies a switch in the plane of cell division (Hardham *et al.* 1980; Brown and Lemmon 1984; Cleary *et al.* 1992a). The cortical arrays apparently function in cell shaping and appear to be essential to cells that are connected to each other by interlocking walls. Cells that function as free cells, such as sporocytes or spores, typically have reticulate or radial microtubule systems that center on the nucleus rather than a distinct system at the cell periphery (for review see Brown and Lemmon 1992a). In pollen tubes, which are unusual in that they exhibit tip growth, the cortical microtubules are net axial or helical in orientation rather than perpendicular to the axis (Lancelle *et al.* 1987; Heslop-Harrison *et al.* 1988; Pierson *et al.* 1989; Steer and Steer 1989; Tiezzi 1991).

The evolution of cortical microtubule systems appears to coincide with the loss of centrioles in green algae. In spite of the fact that cells of the monostromatic species of the charophycean green alga *Coleochaete* divide in two planes resulting in growth that resembles the parenchymatous growth of higher plants, the cortical microtubules remain focused on centrioles after mitosis (Marchant and Pickett-Heaps 1973; Brown and Lemmon 1990a). In the filamentous green alga *Mougeotia*, in which centrioles have been lost, cortical microtubules tend toward hoop-like arrangement (Pickett-Heaps and Wetherbee 1987; Galway and Hardham 1991). Detailed study of the nucleation and organization of hoop-like cortical microtubules in higher plants has led to the suggestion that the cortical system is either intiated at the nucleus and transported to the cortex (Wick 1985; Lloyd 1987) or controlled by MTOCs residing in the cortical cytoplasm especially at the cell edges (Gunning *et al.* 1978; Palevitz 1981; Cleary and Hardham 1988, 1990). Observation of cell division in simple land plants leaves us with the impression that nuclear/perinuclear control of microtubule organization is operative immediately after mitosis and that organizational control is transferred to the cortical cytoplasm during the early stages of interphase. This may involve either sequential activiation of nucleation sites or relocation of the MTOC (Cleary *et al.* 1992a, 1992b).

The cell cycle of plants is unique in having a cytokinetic apparatus consisting of two temporally disjunct cytoskeletal components, the preprophase band (PPB) and the phragmoplast. The PPB develops in interphase and is the first structural indication

that a cell is about to divide. The PPB disappears as the mitotic spindle is organized and the phragmoplast appears as the spindle is dismantled after mitosis. For convenience, we will treat the three as they appear in the cell cycle.

Preprophase band of microtubules. The PPB was first described as a cortical band of microtubules that appears before mitosis in plant cells (Pickett-Heaps and Northcote 1966a, 1966b). Since this discovery nearly thirty years ago, numerous studies have shown that the PPB accurately predicts the plane of cell division in histogenesis (for reviews see Gunning 1982; Wick 1990, 1991). The PPB has been shown to contain actin filaments that are co-aligned with the microtubules (Kakimoto and Shibaoko 1987; Lloyd 1987; Palevitz 1987). There is general agreement that the PPB is a manifestation of cellular polarization in preparation for cell division rather than the determinant of division polarity. Recent studies on the distribution, structure, and function of the PPB indicate that the PPB is instrumental in preparing the parental wall for junction with the expanding cell plate that will complete cytokinesis after mitosis (Mineyuki and Gunning 1990; Sawidis *et al.* 1991). Additionally, there is some evidence that the PPB influences orientation of the spindle axis (Wick 1990, 1991).

Spindle. The mitotic spindle in meristematic cells arises from diffuse polar regions that define the spindle axis which typically lies at right angles to the PPB. Microtubules extending between polar regions and the PPB may represent a mechanism for final alignment of the spindle axis with the future plane of cytokinesis. The shape of the mitotic apparatus ranges from fusiform with pointed poles to barrel-shaped with broad poles. Plant spindles often exhibit numerous regions where subsets of microtubules converge into what have been termed "minipoles" by Schnepf (1984). Variation in the degree of polar consolidation as seen in different stages of the same plant (Hogan 1987), in mutants (Staiger and Cande 1990) and in drug-treated cells (Clayton and Lloyd 1984; Brown and Lemmon 1992d) has led to the concept that targetable factors control polar aggregation.

Phragmoplast. The phragmoplast consists of co-aligned microtubules and actin filaments organized into fusiform bundles bisected by a mid-zone where vesicles are concentrated in the plane of cytokinesis. Fusion of the vesicles results in a cell plate which matures into the new cell wall. The phragmoplast is initiated in an interzonal array of microtubules that proliferates between the two groups of anaphase/telophase chromosomes (e.g Bajer 1968; Gunning 1982; Schopfer and Hepler 1991). As the phragmoplast expands centrifugally, fusion of vesicles in the mid-zone results in deposition of the cell plate that eventually joins with the parental wall at the site previously prepared by the PPB. The tight coordination of phragmoplast expansion and cell plate deposition in vegetative mitosis obscures the separate nature of these distinct components of plant cytokinesis. Typical phragmoplasts can develop and expand normally without the fusion of vesicles into a cell plate (Brown and Lemmon 1991a, 1992d), and in the presence of caffeine, cell plates may grow but fail to fuse with the parental wall and disintegrate (Bonsignore and Hepler 1985). Thus, cytokinesis in organized tissues depends on at least four processes: preparation of the division site by the PPB, transport of vesicles to the division site by the phragmoplast, development of a cell plate by fusion of vesicles, and union of the cell plate with the parental wall.

The Control of Division Plane in Plant Cells

The development of form in plants is directly related to the planes of cell division and to the subsequent growth of daughter cells. Unlike animal cells which may migrate to final position, the walled cells of plants are fixed in position. As a consequence, plant

Microtubules Predict Division Site

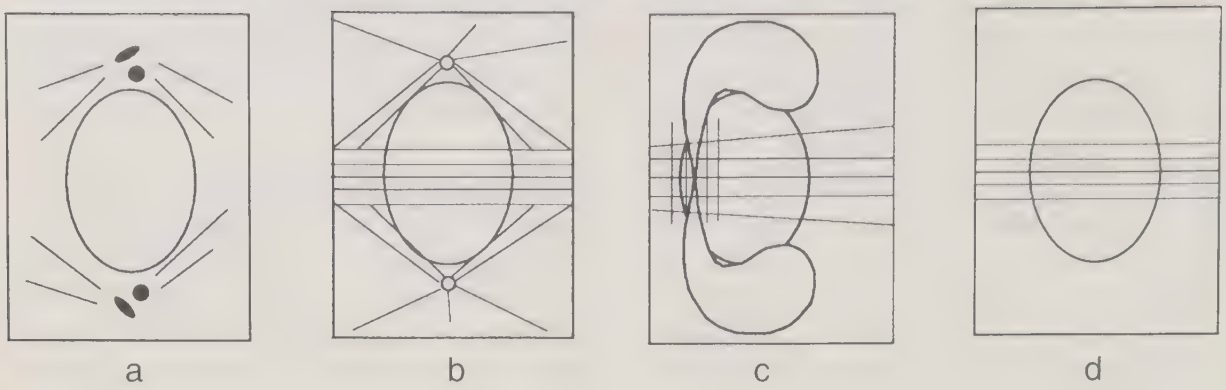


Fig. 1. Diagram showing microtubule arrays that predict division polarity. a. In many algae, astral microtubules emanating from centriolar centrosomes mark poles of the division axis. Intermediate stages occur in bryophytes where centrosomes are absent but microtubules mark the spindle axis slightly in advance of a preprophase band of microtubules (PPB) in the division site. b. In hepatics, microtubules emanating from opposing polar organizers interact in the equatorial region where a PPB forms. c. In hornworts, the axial microtubule system (AMS) associated with the plastid isthmus coexists with an asymmetrical PPB. d. In higher plants, the first microtubules indicative of division polarity in vegetative mitosis are those of the PPB marking the division site.

cells have evolved elaborate mechanisms for the control of the division plane. Since the discovery of the PPB by Pickett-Heaps and Northcote (1966a, 1966b) considerable evidence has been put forth to support the concept that plant cells select the plane of cytokinesis early in preparation for cell division (for review see Gunning 1982; Wick 1990, 1991). Once selected the division site, although structurally unmarked after prophase, controls the final path of the phragmoplast and determines junction of the new wall with parental wall. Mineyuki and Gunning (1990) have proposed that the PPB controls wall placement by governing the localized deposition of insertion and maturation factors into the parental walls. Thus, the control of division plane is, at least indirectly, a function of the PPB in meristematic cell division.

Incorporation of the PPB into the cytokinetic apparatus appears to have been coincidental with the loss of centrioles and with development of a cortical system of microtubules underlying the wall. PPBs are lacking in the advanced green alga *Coleochaete* where microtubules are organized at centrosomes (Marchant and Pickett-Heaps 1973; Brown and Lemmon 1990a), but structures resembling PPBs have been reported in the filamentous green alga *Mougeotia* (Galway and Hardham 1991). Although the filamentous growth form seems more primitive than the two dimensional growth of *Coleochaete, Mougeotia* is more like higher plants in that centrioles are lacking and in the presence of a cortical array of microtubules in interphase. Considerably more information will be needed before we can speculate on the steps that led to establishment of the PPB into the cytokinetic apparatus of land plants, but it would seem certain that its evolution began in putative algal ancestors and became firmly established in the early land plants. Possible stages in the evolution of microtubule systems associated with division polarity are shown in Fig. 1. Some clues to the temporal shift away from establishment of spindle poles toward prior establishment of the division site marked by a PPB are to be found in the bryophytes.

PPBs are components of cell division in organized vegetative tissues of both generations of bryophytes, having been reported in gametophytes of liverworts (Fowke and Pickett-Heaps 1978; Apostolakos and Galatis 1985; Brown and Lemmon 1990b), sporophytes of hornworts (Brown and Lemmon

1988b), and in both the sporophytes (Sack and Paolillo 1985) and gametophytes of mosses (Schnepf 1973; Schmiedel *et al.* 1981; Doonan *et al.* 1987). However, certain phases of complex protonematal growth of mosses occurs in the absence of PPBs. PPBs are lacking in tip growth of the protonemal filament and in the development of side branches (Doonan *et al.* 1987; Doonan and Duckett 1988; Doonan 1991), but are present when leafy gametophores are developed (Doonan *et al.* 1987) and when specialized Tmema cells are initiated (Sawidis *et al.* 1991). Recent studies of Tmema cells in the Funariales support the concept that the PPB prepares the parental wall for junction with the new wall. Tmema cells are initiated by a highly asymmetrical division and result in fragmentation. Unlike other protonemal cells that exhibit tip growth and lack PPBs, the Tmema mother cell undergoes elongation growth similar to cell elongation in meristems of tracheophytes. Sawidis *et al.* (1991) proposed that the PPB directs deposition of a layer of cellulose which supports attachment of the Tmema cell to the filament during elongation by cell wall expansion, a process unlike other cells in the protonema which exhibit tip growth and lack PPBs. Comparable information on PPBs in germling stages of hepatics would be useful particularly in view of the fact that germination patterns and early growth forms vary with species as well as with changes in growth media. Since similar mechanisms of suppression/desuppression are known to be operative in both hepatics and flowering plants (for review see Basile and Basile, this volume), the hepatics offer potential for incisive research on the functional relationship of wall components to underlying microtubule systems and their respective roles in the developmental pathways of plants.

PPBs are absent during the mitoses which give rise to sporocytes in the archesporial tissues of bryophytes (Busby and Gunning 1988a, 1988b; Gambardella and Alfano 1990; Brown and Lemmon 1992b). In hornworts, PPBs are present in dividing cells of the intercalary meristem until just before sporocytes are differentiated (Brown and Lemmon 1988b). In the hepatic *Monoclea*, PPBs are absent in the four or five rounds of mitosis that result in the proliferation of single cells into 16, 32 or more cells which differentiate into sporocytes (Brown and Lemmon 1992b). In mosses, PPBs are apparently absent during the several mitoses responsible for proliferation of archesporial cells prior to their enlargement into sporocytes (Gunning and Busby 1988a, 1988b; Gambardella and Alfano 1990). This is in keeping with the situation in higher plants where PPBs are absent in the reproductive lineage that gives rise to microspores by meiosis and then to sperm by mitotic divisions (Hogan 1987; Brown and Lemmon 1992a). Since PPBs regularly are a part of vegetative mitosis, we assume that the absence of PPBs marks the entrance of a cell into a developmental pathway leading to free-living single cells that are no longer part of an organized tissue.

Table 1. Structural Evidence of a Division Site

1. Phragmosome
2. Preprophase band of microtubules
3. Cytoplasmic infurrowing/wall ingrowth
4. Organelle band
5. Morphogenetic plastid migration *
6. Axial microtubule system *
7. Procytokinetic plate *
8. Quadripolar microtubule system *

* Monoplastidic cells only

Comparative studies of cell division in the lower land plants have produced additional evidence that plant cells select the future plane of cytokinesis before the nucleus divides. These markers for establishment of division polarity in cells with and without PPBs help to understand the mechanisms by which plant cells achieve precise division patterns. A list of structures that predict the plane of division is shown in Table 1. Those marked with an asterisk are known only from monoplastidic cell division. In

many of the simple plants, cell division (mitosis and/or meiosis) occurs in cells that contain a single plastid which migrates in response to the establishment of division polarity (for review see Brown and Lemmon 1990a). Monoplastidic cell division is known to occur in all the bryophyte groups, although it appears to be rare in hepatics (Brown and Lemmon 1990a, 1992b). We consider monoplastidy to be a primitive character since it is the typical condition in green algae including the charophycean algae, common in dividing cells of bryophytes and lycopsids, extremely rare in ferns, and unknown in seed plants.

Predivision behavior of the plastid is as predictive of division polarity as is the PPB. We use the term "morphogenetic plastid migration" in reference to the regular pattern of plastid migration associated with monoplastidic cell division. Morphogenetic plastid migration is predictive of the future plane of cytokinesis in all classes of division. The single plastid consistently comes to lie in a position parallel to the future spindle axis with its midregion intersecting the division site. In vegetative mitosis where a PPB is formed, morphogenetic plastid migration may occur either prior to development of the PPB or concommitant with its appearance.

The mechanism by which the large plastid moves into proper position for apportionment into daughter cells is largely unknown. It seems certain that microtubules play a significant role. Sporocytes exhibit a complex system of microtubules associated with plastids as they achieve polar arrangement (Brown and Lemmon 1982, 1987a, 1987b; Busby and Gunning 1988a, 1988b), but in mitotic cells plastid migration more typically occurs in the absence of elaborate microtubule arrays (Brown and Lemmon 1990a; Cleary *et al.* 1992a, 1992b). In sporocytes of the moss *Funaria hygrometrica* the plastid can divide, but is unable to migrate, in the continued presence of the microtubule poison colchicine (Busby and Gunning 1989).

Treatment with oryzalin demonstrated that microtubules are required for proper positioning of the plastids, but once a tetrahedral arrangement is acheived microtubules are no longer necessary (Busby and Gunning 1989). Likewise, the association of actin with migrating plastids has yet to be demonstrated conclusively, and the relationship of morphogenetic plastid migration to photostimulated plastid migration, which is driven by actin (Haupt 1982; Witztum and Parthasarathy 1985; Grolig and Wagner 1987), has not been elucidated. While it seems likely that the mechanisms of moving the large organelle through the cytoplasm may have much in common in the two phenomena, the triggers would be expected to be different. Although we have not yet conducted critical experiments to test the effect of different light stimuli on dividing cells, we have no reason to suspect that morphogenetic plastid migration is sensitive to these triggers. Certainly, this line of research could be expected to provide information on the underlying signal pathways responsible for plastid migration in plant cells as well as on the actual mechansims of organelle migration in response to cell polarity.

Another outstanding example of the predivision establishment of polarity in lower land plants is the quadrilobing of sporocytes before meiotic prophase (for review see Brown and Lemmon 1988a, 1990c, 1991b). Cytoplasmic lobing, which predicts the arrangement of the spore tetrad, occurs in meiosis of all bryophytes except for members of Marchantiidae (Schuster 1966, 1984; Brown and Lemmon 1988a, 1990c, 1991b). It is within the hepatics that the most variation is seen with regard to quadrilobing and monoplastidy. All of the mosses and hornworts studied exhibit both lobing and monoplastidy, with plastid division and migration resulting in a single plastid in each of the four lobes that define domains of the future spores. The vast majority of hepatics are polyplastidic and monoplastidy has been discovered only recently in the sporocytes of three disparate hepatics; *Monoclea gottscheii, Blasia pusilla,* and

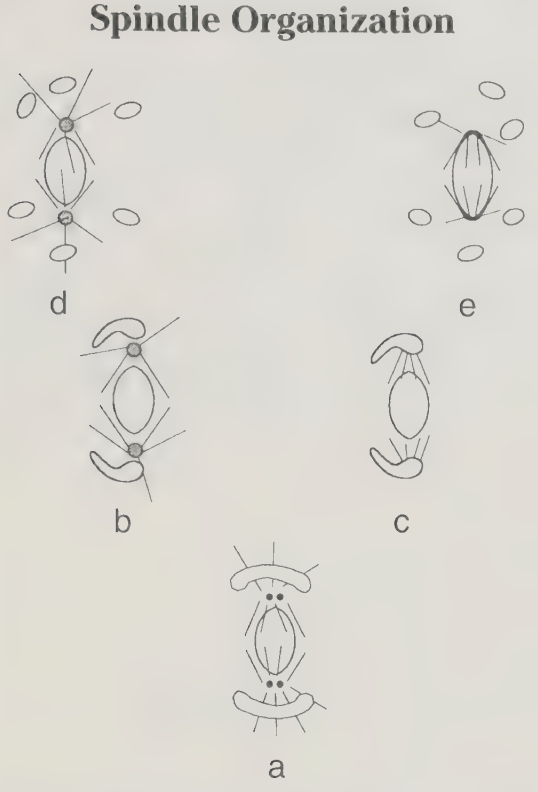


Fig. 2. Possible stages in evolution of the diffuse mitotic spindle of higher plants from the astral spindle of algae. a. The astral spindle of the alga *Coleochaete* emanates from polar centrosomes between the nucleus and a daughter plastid at each pole. b. The spindle of the hepatic *Monoclea* emanates from acentriolar polar organizers between the nucleus and a daughter plastid at each pole. c. The spindle of mosses, hornworts, and lycopsids emanates from daughter plastids at the poles. d. The spindle of the polyplastidic hepatics emanates from polar organizers; numerous plastids cluster in the polar regions. e. The diffuse spindle of higher plants is organized in the perinuclear area; numerous plastids are more randomly distributed.

Haplomitrium blumei (Brown and Lemmon 1992b, unpublished). Although morphogenetic plastid migration is usually coordinated with cytoplasmic lobing, it apparently does not control it. Lobing is initiated prior to plastid migration in the moss *Atrichum* (Brown and Lemmon 1987a) and extreme lobing occurs in the polyplastidic sporocytes of hepatics.

The Plant MTOC and Spindle Ontogeny

Study of variation in the way that spindles are organized in bryophytes provides some clues to the evolution of the diffuse perinuclear MTOC characteristic of higher plants (Fig. 2). While true centrosomal spindle organization in land plants is known only in spermatogenesis after centrioles appear de novo in the penultimate division (Robbins 1984; Renzaglia and Duckett 1988), there is evidence of discrete polar organizers in cell division of hepatics which provides some continuity with the centrosomes of algae. In the hepatic *Reboulia*, opposing systems of microtubules emanating from POs, which appear at opposite poles of an elongated nucleus, give rise to the half-spindles (Brown and Lemmon 1990b). A PPB forms in the region of interaction between the opposing arrays. The sequence of events appears to be an intermediate stage between algae where spindle poles are marked by migration of centrioles (e.g. *Coleochaete*) and higher plants where the PPB marks the division site before the spindle poles are defined (Fig. 2). When microtubules are stained with techniques of immunofluorescence, the PO appears as an unstained area from which microtubules radiate in an astral array. TEM reveals that the PO itself consists of an electron dense area crowded with endoplasmic reticulum (Fowke and Pickett-Heaps 1978; Steer 1984; Apostolakos and Galatis 1985; Brown and Lemmon 1992b); more concentrated, but otherwise not unlike, polar areas in certain higher plants (Esau and Gill 1969; Ryan 1984; Hepler *et al.* 1990; Brown and Lemmon 1992c)

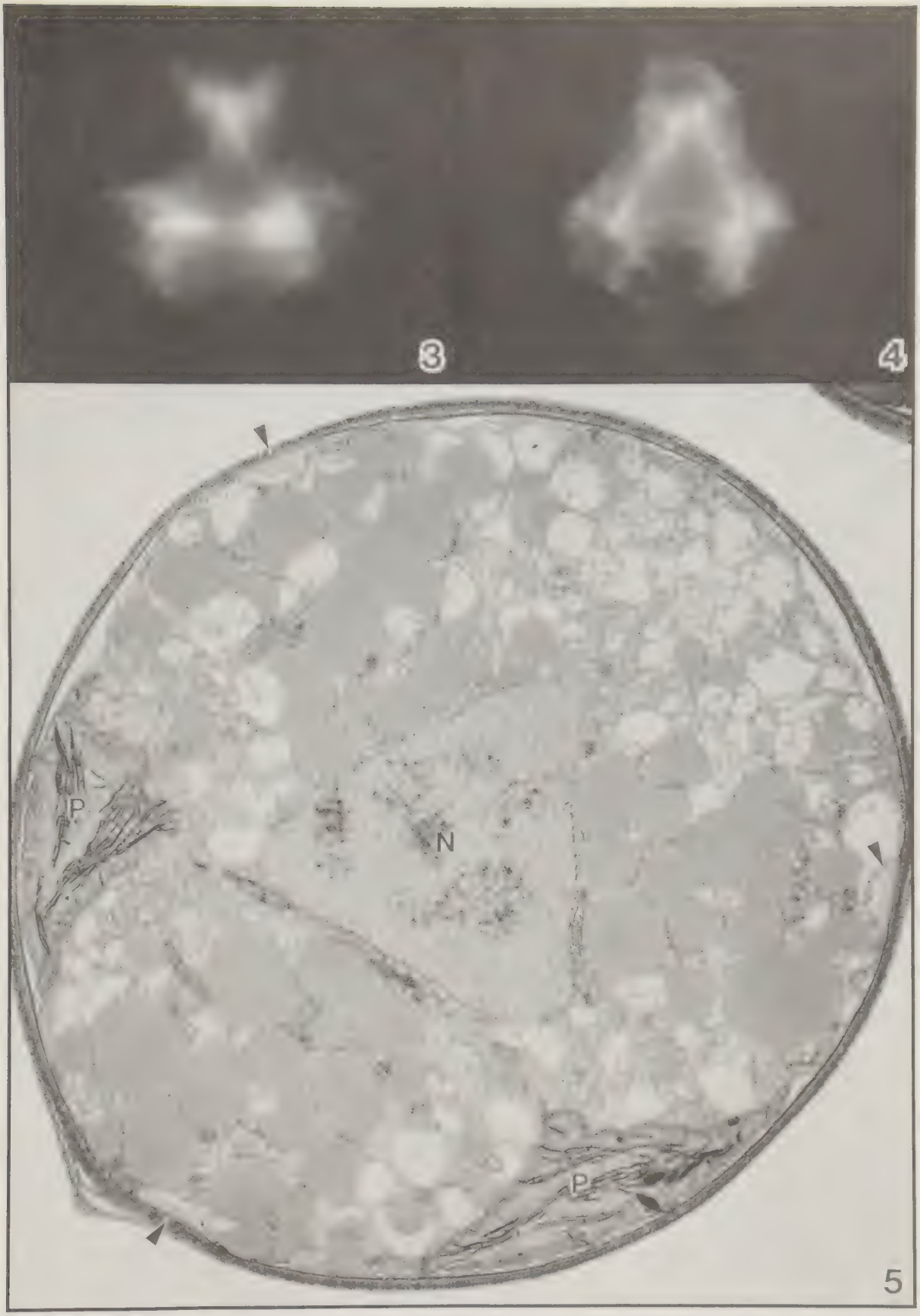
In monoplastidic cell division of bryophytes and lycopsids, the MTOC material is typically associated with the plastids (for review see Brown and Lemmon 1990a; Cleary *et al.* 1992b). Monoplastidic cells do not show the perinuclear distribution of tubulin/microtubules reported in early mitosis of flowering plants (Wick and Duniec 1983, 1984). The close association of MTOC material with the plastid envelope is supported by the reappearance of microtubules in association with the plastid in sporocytes of *Funaria* recovering from drug induced microtubule depolymerization (Busby and Gunning 1989). The only exception to this evolutionary step away from

centrosomal spindle ontogeny is in the few aforementioned hepatics with monoplastidic cell division. In *Monoclea gottschei*, the plastid divides and the daughter plastids migrate to the future spindle poles, but the spindle microtubules emanate from discrete POs located between the nucleus and plastids (Brown and Lemmon 1992b). In all other land plants with monoplastidic cell division (mosses, hornworts, and lycopsids), the microtubules appear to emanate from the plastid envelope (Brown and Lemmon 1990a; Cleary *et al.* 1992b).The mitotic spindle develops from two opposing plastid-based sets of microtubules and the meiotic spindle arises from four plastid-based sets of microtubules. This quadripolar microtubule system (QMS) that develops in meiotic prophase becomes bipolar by metaphase I, by either of two methods (Brown and Lemmon 1991b). In bryopsid mosses (Brown and Lemmon 1987a, 1987b; Busby and Gunning 1988a, b) and hornworts (Brown and Lemmon 1990d); the MTOCs move away from the tetrahedrally positioned plastids to converge in pairs upon the spindle axis between plastids. The other method is seen in megasporogenesis of *Isoetes* where the MTOC material remains associated with the plastids and the plastids themselves move to the spindle axis (Brown and Lemmon 1989).

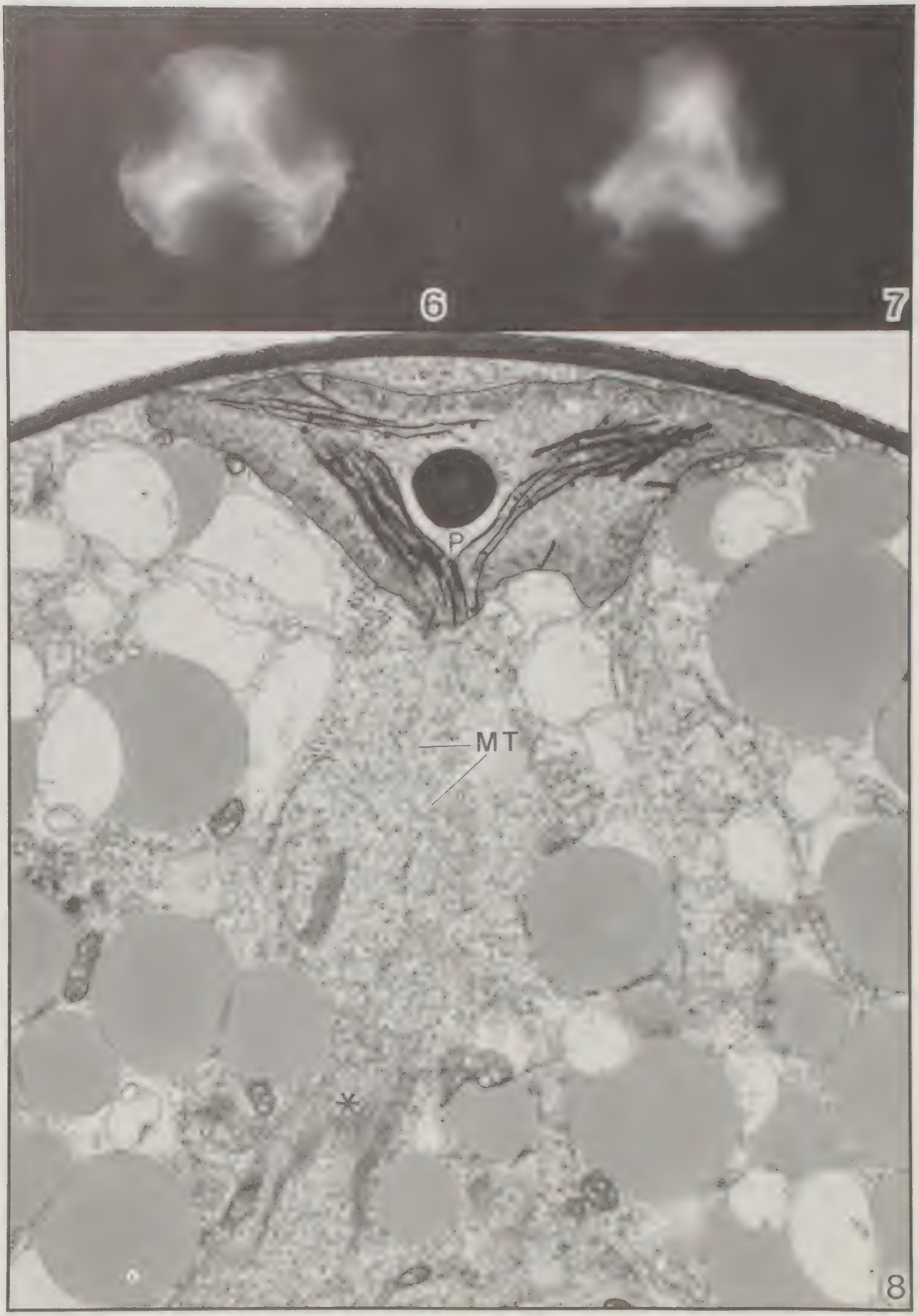
An interesting variation in meiotic spindle maturation is seen in *Sphagnum* (Figs. 3–11). Two divisions of the single plastid result in four plastids which become tetrahedrally positioned in the sporocyte. Microtubules emanating from the plastids converge on the central nucleus and encage it in a QMS (Figs. 3–8), as is typical of spindle ontogeny of bryopsid mosses. In *Sphagnum*, however, the MTOCs neither leave the plastids nor do the plastids migrate to the spindle axis. This results in a most unusual metaphase spindle that is triangular in shape (Figs. 9–11) when viewed from any side. Each pole stretches between a pair of plastids and the two poles are at right angles to each other. Chromosomes are distributed into groups that stretch between pairs of plastids and the telophase nuclei are correspondingly long and slender.

Comparative studies of mitosis and meiosis in the hornworts *Phaeoceros* and *Notothylas* have been useful in providing the basis for a model to show the relationship of plastid behavior and spindle ontogeny in mitosis and meiosis (Brown and Lemmon 1988b, 1990d). The hornworts are unique in that there is a microtubule system associated with plastid division. This system, which lies parallel to the long axis of the plastid, is designated the axial microtubule system (AMS) (Brown and Lemmon 1988b). In mitotic cells the AMS is initiated slightly in advance of the PPB, and the two mutually perpendicular systems coexist during preprophase/prophase (Figs. 12 and 13). The AMS gives rise to the mitotic spindle by elongating into two opposing sets of microtubules (Fig. 12). Thus, as in the hepatic *Reboulia*, the spindle axis appears to be established independently of the PPB marking the division site. In hornworts, division polarity is marked first by position of the plastid, followed by the AMS, and then by the PPB. This is in contrast to the situation in higher plants where the PPB is the first indication of division polarity. Spindle ontogeny in hornworts appears to involve poleward migration of MTOC material along the plastids in a pattern similar to the poleward migration of centrosomes. The spindle of hornworts develops similarly, and is even more conspicuously asymmetrical in the early stages than is the spindle developed from astral microtubules associated with poleward bound centrosomes in algal and animal cells

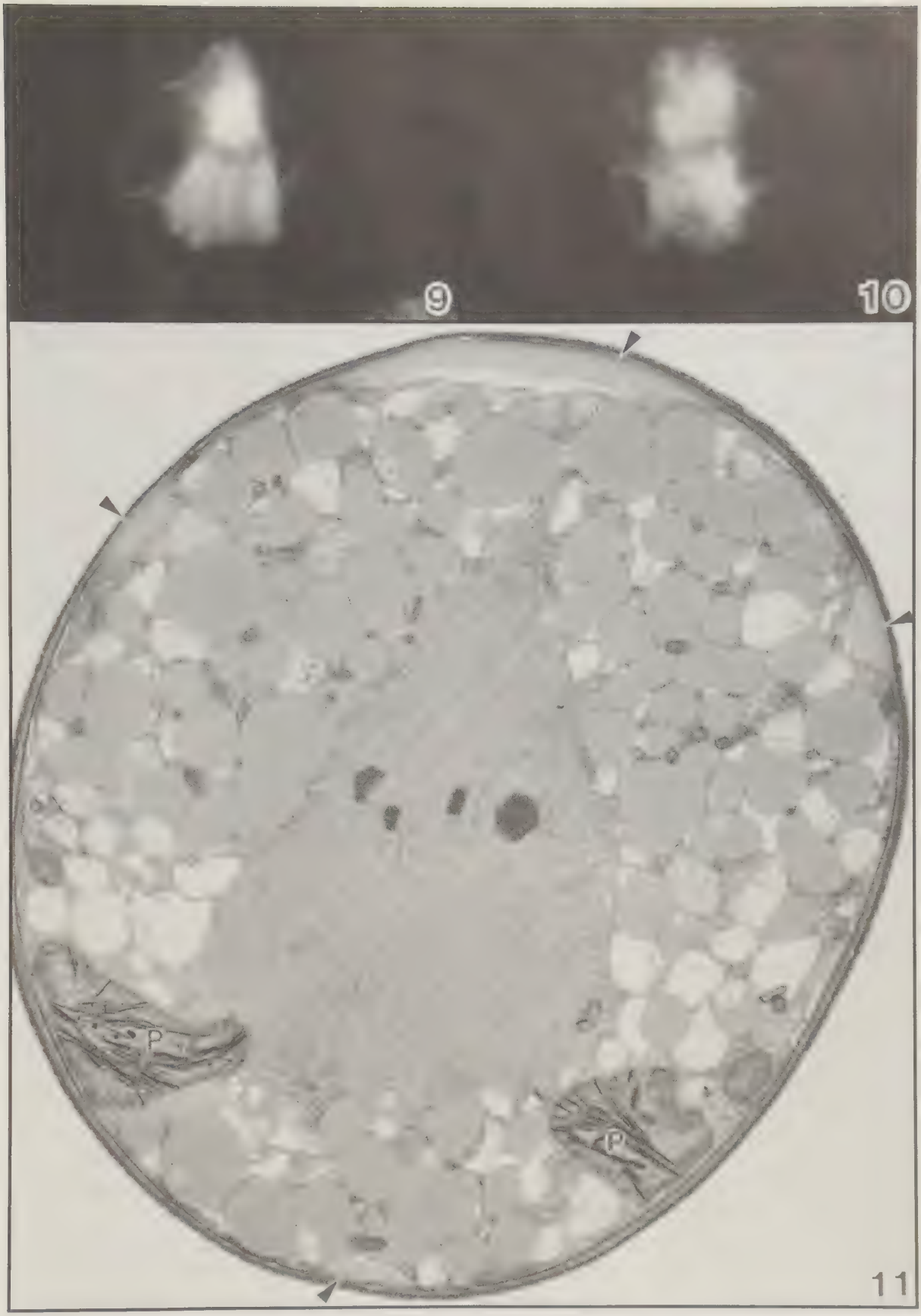
Hornworts provide a convenient system where plastid behavior and spindle ontogeny in meiosis can be compared to that in mitosis (Figs. 13–16). In the first plastid division of meiotic prophase, the AMS elongates somewhat but does not give rise to a spindle (Fig. 14). In the second plastid division, an AMS appears in the midregion of each of the plastids (Fig. 15), which rotate so that they lie at right angles to each other. This position places the four plastid tips in



Figs. 3–5. Meiotic prophase in sporocytes of *Sphagnum*. 3–4. Microtubules stained by indirect immunofluorescence shown at two levels of focus. Microtubules emanating from four tetrahedrally arranged plastids encage the central nucleus in a quadripolar microtubule system (QMS). x2,000. 5. TEM of a near median section through a sporocyte showing that the nucleus (N) is distorted toward the plastids (P). The two plastids that are out of the plane of section would lie at right angles to the pair of plastids shown in the micrograph. Wall ingrowths (arrowheads) mark the future cleavage planes. x5,000.



Figs. 6–8. Spindle development in sporocytes of *Sphagnum*. 6–7. Microtubules stained by indirect immunofluorescence. x2,000. 6. Microtubules emanating from the four tetrahedrally positioned plastids become organized into columns encaging the nucleus which is distorted into the shape of a tetrahedron. 7. These microtubules give rise to a quadripolar spindle. 8. TEM showing detail of the developing spindle. Numerous microtubules (MT) extend from the plastid (P) to envelop the distended nucleus in a highly ordered array (*). x18,700.



Figs. 9–11. Metaphase I in sporocytes of *Sphagnum*. 9–10. Microtubules stained by indirect immunofluorescence; chromosomes in the equatorial region are unstained. The metaphase I spindle in side view appears triangular in shape as it remains anchored at the four tetrahedrally positioned plastids. The two polar regions are broad and at right angles to each other. x1,670. 9. The lower polar region is seen in face view while the upper polar region is seen edge on. 10. Quadripolarity of the metaphase spindle can be seen in this view. 11. TEM of a median section through a sporocyte oriented similar to that shown in Fig. 9. The two half spindles are at right angles to one another. In face view (lower portion of micrograph) the spindle can be seen to be anchored between a pair of plastids (P). In edge view (upper portion of micrograph) the pair of plastids are out of the plane of section. Wall ingrowths (arrowheads) mark the future cleavage planes. x5,400.

Spindle Organization

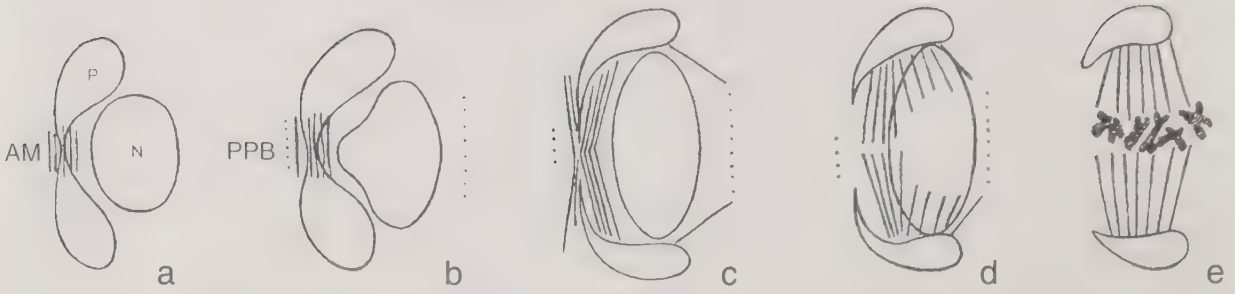


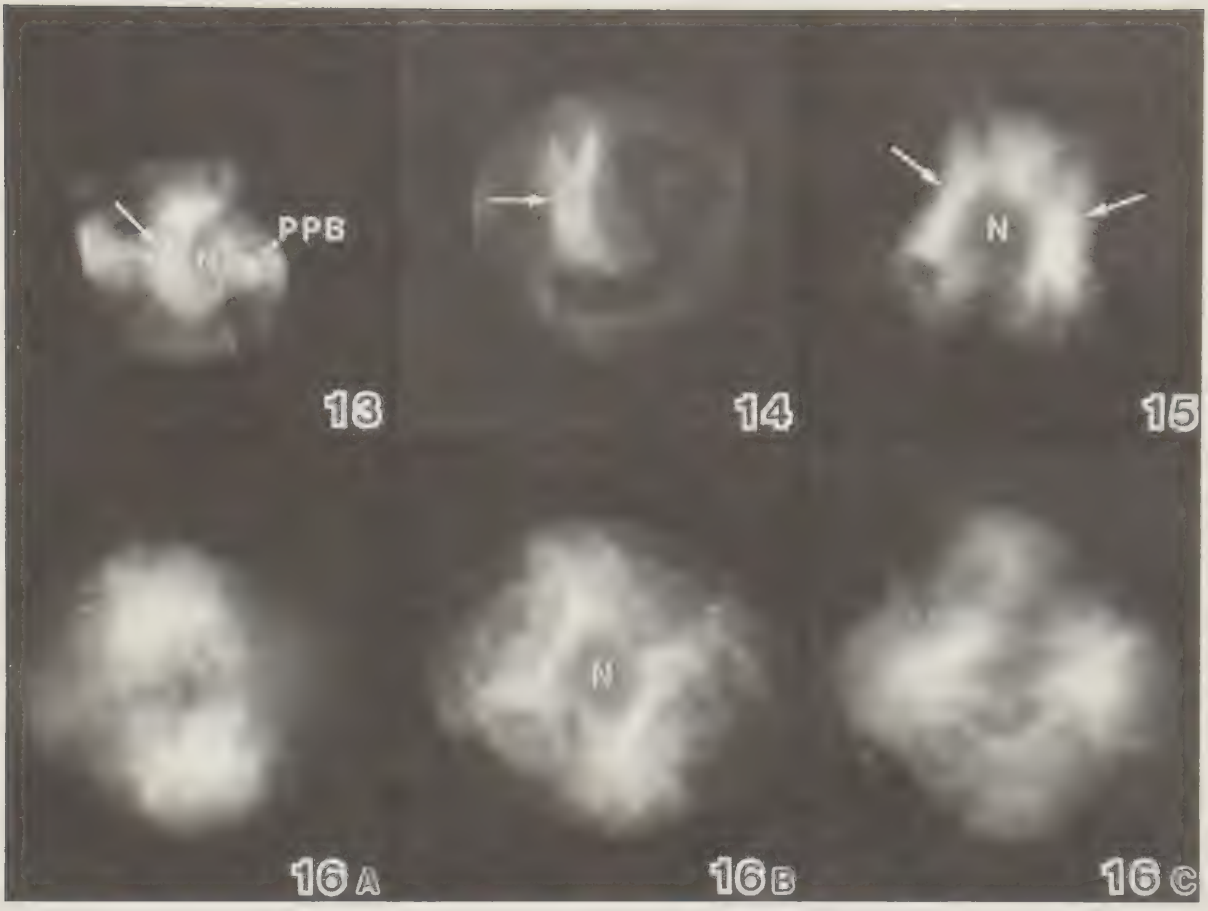
Fig. 12. Diagram of microtubule systems associated with mitosis in hornworts. a. In preprophase, an axial microtubule system (AMS) lies parallel to the long axis of the plastid and marks the future spindle axis. b. An asymmetrical PPB (shown in cross section) that is narrow over the plastid and flares out over the nucleus, girdles the cell in the equatorial region. c. The AMS elongates and interacts with the microtubules of the PPB. d. The AMS develops into two half-spindles focused on the plastid tips. e. By metaphase, the PPB has disappeared and the spindle is symmetrical.

tetrahedral position and predicts the future arrangement of spores in the tetrad. The two AMSs, which lie on either side of the undivided nucleus, elongate and develop into opposing sets of microtubules focused on the plastid tips. These four sets of microtubules develop into a single QMS. The QMS is made symmetrical as the second plastid division is completed, and microtubules radiating from the four plastids encage the central nucleus in four interacting cones of microtubules (Fig. 16). Thus, we interpret the meiotic spindle as a merger of two mitotic spindles, the axis of which passes between the pairs of poles. This is in keeping with observations on spindle ontogeny in microsporogenesis of *Selaginella* where the microtubule arrays which develop parallel to each of the two plastids flanking the nucleus merge into a single meiotic spindle, the axis of which lies between the late dividing plastids (Brown and Lemmon 1991c). In meiotic prophase of mosses, the plastids typically separate and the four plastid-based arrays interact to form the QMS encaging the nucleus. In *Funaria*, however, the microtubule arrays parallel to each of two late dividing plastids (primary arrays) are distinguishable from those which subsequently emanate from the plastid tips (secondary arrays) (Busby and Gunning 1988a, 1988b). This indicates similarity in the ontogeny of the QMS in mosses and hornworts.

All of the above cited examples of microtubule organizing material associating with, and leaving, plastid membranes suggests that the plant MTOC is a fluid entity capable of responding to changes in polarity associated with morphogenesis. Another outstanding example of MTOC migration has been documented in development of the spore aperture in the moss *Tetraphis* (Brown and Lemmon 1983). As in all mosses, the second division spindles terminate in pairs of plastids and each spore nucleus is lodged proximal to a plastid. The plastid associated MTOC material becomes concentrated on the distal side of the plastid and numerous microtubules radiate from it to underlie the developing exine. Once the distal surface of the spore is covered with exine, the MTOC and plastid move around one side of the nucleus. The MTOC leaves the plastid and becomes positioned under the proximal surface where it is associated with the development of an aperture. Since the tetrad is still contained within the original sporocyte wall at this time, and the moving MTOC is the center of numerous microtubules, it is easy to follow the path of migration from distal to proximal surface.

The Phragmoplast and Cytokinesis

Cytokinesis in plant cells results from new wall formation by fusion of vesicles concentrated in the midzone of the phragmoplast.



Figs. 13–16. Comparison of microtubule systems at the onset of mitosis and meiosis in hornworts. Microtubules stained by indirect immunofluorescence. x1,500. 13. Preprophase of mitosis. The AMS (arrow), which lies parallel to the isthmus of the elongating plastid, will give rise to the spindle. The PPB marking the equatorial region lies at right angles to the AMS. N, nucleus. 14–16. Development of the quadripolar microtubule system (QMS) from two AMSs in meiotic prophase. No PPBs are formed in meiotic cells. 14. The AMS (arrow) associated with the first plastid division does not develop into a spindle (Compare with Fig. 12.). 15. An AMS forms in the isthmus region of each of the daughter plastids (arrows) at second plastid division. N, nucleus. 16. Three levels of focus showing development of the plastid-based QMS after second plastid division. Each AMS elongates into two opposing sets of microtubules focused on the plastid tips. The two plastids rotate so that the four tips are in tetrahedral position. 16a. Upper surface view showing two cones of microtubules emanating from a pair of plastids. 16b. Optical mid-section showing the four cones of plastid-based microtubules encaging the nucleus (N). 16c. Lower surface view showing the two cones of microtubules emanating from the pair of plastids at right angles to the pair shown in 16a.

Thus, the phragmoplast appears to be the instrument responsible for vesicle transport and alignment (e.g. Gunning 1982; Schopfer and Hepler 1991). Although the phragmoplast is remarkably uniform in all land plants, it can be initiated in a wide variety of microtubule arrays. In the green alga *Coleochaete*, the phragmoplast is initiated at the interface of opposing sets of microtubules emanating from centrioles at the telophase poles (Brown and Lemmon, unpublished). In monoplastidic mitosis, the phragmoplast is initiated in the interface of microtubules emanating from the polar plastids as well as from the chromosomes/telophase nuclei (Cleary *et al.*1992b). In

simultaneous cytokinesis following meiosis, phragmoplasts develop in the interzonal microtubule arrays between sister nuclei, and also in secondary arrays interconnecting non-sister nuclei (for review see Brown and Lemmon 1992a). Similarly, phragmoplasts among nonsister nuclei develop in cellularization of endosperm (van Lammeren 1988). In all cases, proliferation and reorganization of microtubules triggered at the interfaces of opposing sets of microtubules results in the complex and highly regular phragmoplast.

The phragmoplast is a cytoskeletal structure that has no counterpart in animal cells. Cytokinesis in animal cells occurs centripetally by infurrowing of the cytoplasm and the only microtubules associated with the process are organized into a midbody that appears to hold the daughter nuclei apart (Euteneuer and McIntosh 1980). Since the midbody arises in the interzone between sister nuclei, it is somewhat analogous to the interzonal array in plant cells. However, no structure resembling a centrifugally expanding phragmoplast (made up of fusiform bundles bisected by a dark line) has ever been shown to develop in the interface of the opposing arrays that make up the midbody. Likewise, cytoplasmic domains defined by microtubules occur in coenocytic cells during development of *Drosophila* larvae (Warn 1986) and *Acetabularia* cysts (Menzel 1986), but no higher level of structural complexity is triggered in the interfaces. The tendency for plant cells that are in certain stages of the life cycle to develop phragmoplasts wherever opposing arrays interact emphasizes a basic difference in the way that plants control the cycling and organization of tubulin (Bajer and Mole-Bajer 1986).

It seems that the coordination of cell plate deposition with phragmoplast expansion occurred early in the evolution of the land plant form. There are examples of phragmoplasts in algal cytokinesis that suggest intermediate stages. For instance, in the filamentous green alga *Spirogyra* (Fowke and Pickett-Heaps 1969) and *Mougeotia* (Pickett-Heaps and Wetherbee 1987; Galway and Hardham 1991), cytokinesis by infurrowing of the cytoplasm is completed by vesicle fusion in a phragmoplast-like structure restricted to the interzonal region. Cytokinesis in the discoid thallus of the higher green alga *Coleochaete scutata* proceeds differently in the two types of divisions (Marchant and Pickett-Heaps 1973). In radial divisions (those perpendicular to the circumference of the thallus), cytokinesis is like that in land plants. Fusion of vesicles in association with centrifugal expansion of a typical phragmoplast completes cytokinesis except for slight ingrowth of the parental wall particularly on the outer edge of the thallus. In circumferential divisions, however, cytokinesis is quite different in spite of the presence of a phragmoplast. Cytokinesis is completely centripetal and microtubules remain in the central portion of the cytoplasm as it is finally separated in an irregular twisting fashion. Furthermore, it seems that the centripetal vesicle fusion is not in the mid-zone of the phragmoplast but rather more at the interior tips of the fusiform bundles (Brown and Lemmon unpublished). This division is unequal; the outer cell, which will divide again to increase the cell file, is much larger than the interior cell. Perhaps conflicting or imperfectly developed cytokinetic pathways are responsible. At any rate, we have clear evidence of the phragmoplast triggered into formation together with failure of vesicles to fuse in the midzone resulting in a modified pattern of cytokinesis.

The study of meiotic cytokinesis in bryophytes was instrumental in conceptualizing the cytoplasmic domain model for the control of division plane in cells lacking PPBs (Brown and Lemmon 1992a). In precociously lobed sporocytes, cytoplasmic domains of the future spores are clearly marked by radial microtubule systems emanating from the tetrad poles in the lobes prior to meiosis. After meiosis, cytokinesis occurs along the predetermined cleavage planes. When microsporocytes of higher plants undergo simultaneous cytokinesis,

the spore domains are defined by postmeiotic arrays of microtubules which radiate from the four nuclei (for review see Brown and Lemmon 1991d, 1992a). Thus, the sporocytes of lower plants (except those of the marchantioid hepatics) are committed to quadripartitioning by the same mechanism as are those of higher plants, but at a different time in the meiotic cell cycle. This indicates a complex pathway that can be temporally altered, upon which a higher level of control can be superposed as seems to be the case in cells with PPBs. Investigation of the factors responsible for suppression of phragmoplast formation during certain phases of the cell cycle would appear to be a productive line of research that could lead to better understanding of cytokinesis.

In summary, the extant relics of the early land flora represent a rich source of diversity that holds clues to the evolution of the highly specialized microtubule systems that characterize cell division and the development of form in higher plants. Much research is needed and comparative studies will continue to add pieces to the puzzle of evolutionary relationships of land plants.

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The role and control of the place-dependent suppression of cell division in plant morphogenesis and phylogeny

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BASILE, D.V. AND M. R. BASILE (Department of Biological Sciences, Lehman College of The City University of New York, Bronx, NY 10468). The role and control of the place-dependent suppression of cell division in plant morphogenesis and phylogeny. Mem. Torrey Bot. Club 25(1): 63–84. – 1993. The perceived roles for place-dependent suppression of cell division in morphogenesis and phylogeny derives from the premise that once cell proliferation starts in a developing plant or plant part, there must be some mechanism to suppress these growth processes at specific times and in specific places, else plants or their parts would have no predictable size or shape. Since the beginning stages of development of many plants or plant parts are virtually indistinguishable, the hypothesis, which logically follows is, "that many if not most of the differences by which we distinguish the form changes that occur during the stages of ontogeny and the course of phylogeny are due to differences with respect to the time and place cell proliferation and/or enlargement is suppressed." We propose three important roles for place-dependent suppression in plant morphogenesis and phylogeny. The first role is in establishing apical meristematic regions in the transition from diffuse to apical growth that occurs during the early stages of ontogeny in all extant land plants manifesting apical growth. This role was presumably established early in the phylogenetic origin of embryophytes from their pre-embryophytic ancestors. The second role of localised suppression is to stop the further development of specific organ primordia after they are initiated. As a consequence there is an apparent loss of either the number or kinds of parts laid down in meristems thereby resulting in the process referred to as "reductive" or "regressive" evolution. An indirect effect of a selective suppression of primordia development in meristems may be a change in distribution of tensile and compressive forces distributed through both the apoplast and symplast of the meristems and a concommitant change in cell division patterns. The third role is the modulation of size and shape of organs having a determinate mode of development, as indicated in our starting premise, that suppression of proliferation generates form.

One means by which spacial regulation of suppression appears to be regulated is through the interrelated actions of auxin, ethylene and certain arabinogalactan proteins (AGPs). AGPs are a class of hydroxyproline-containing glycoproteins associated with the plasma membranes and extracellular matrices of all intact plant cells. Auxin translocation is probably the principle determinant of where (place) suppression occurs. The onset of ethylene synthesis, whether triggered by auxin or some other factor(s), is probably most responsible for determining when (time) suppression occurs. One or more AGPs probably determine how or whether suppression is maintained in potentially proliferating cells.

Key Words: place-dependent suppression, desuppression, morphogenesis and phylogeny, reductive evolution, hydroxyproline proteins, arabinogalactan proteins, auxin, ethylene, cell proliferation.

The capacity for cells to divide and thereby reproduce is one of the defining properties and processes of life. In the approximately three billion years between the evolution of cellular life and the origin of the earliest land plants (Metaphyta or Embryophyta) in the Middle Silurian, this capacity has apparently continued to be "expressed" in all or most pre-embryophyte cell lineages. In most extant representatives of the putative pre-embryophytes (Chlorophytes), this capacity is still expressed by most vegetative/somatic cells during the course of ontogeny.

Although it is impossible to demonstrate, it is reasonable to infer that in the earliest known embryophytes, *Cooksonia spp.*, the capacity to divide was "suppressed" in most somatic cells. This inference is based on what is presently known about morpho-

genetic processes that result in strict dichotomous branching in extant plants such as *Psilotum* where all proliferative cell divisions in the developing stem axis are restricted to a relatively small population of cells at the apex of each developing axis. In the great majority of living cells that make up the body of these plants, proliferative cell division is completely suppressed. The inferential evidence strongly suggests that the pattern of morphogenesis in *Cooksonia* and, indeed, all taxa assigned to the Rhyniophyta, developed according to a common morphogenetic pattern: cell proliferation "expressed" at the very apices vs cell proliferation "suppressed" everywhere else (Fig. 1).

This observation brings us to propose that the earliest and one of the most critical roles for place-dependent suppression of cell proliferation was to restrict proliferative cell divisions to the extreme apex of developing axes by virtue of suppressing all growth below the apices. Since strict apical, meristematic development is not characteristic of pre-embryophytes, a critical "event" for the origin and diversification of embryophytes from their presumed pre-embryophyte ancestors was the establishment of a regulatory system whereby one subpopulation of cells could act to suppress further division in the remaining, adjacent population. This is essentially the course of events during the early ontogeny of any extant plant (with the exception of the sporophytes of liverworts and the gametophytes of heterosporous taxa in which ontogeny proceeds from zygote or spore, respectively, to reproductive maturity without passing through an apical growth stage). Whether starting from a zygote or spore, early development ot most plants usually proceeds through an early "embryonic" stage whereby the cells of the plant body are generated by divisions occurring in a more or less diffuse pattern throughout. After a relatively short period of diffuse cell divisons, one or more subpopulations continue to express the innate capacity to divide while in the others this innate capacity is suppressed (Fig. 2). There is no question that place-dependent (subapical) suppression has a primary role in the ontogenetic origin of apical growth in all groups of extant plants. The only thing still open to question is whether the place-dependent suppression that initiates apical growth during ontogeny essentially "recapitulates" the same stage change that occurred early in Embryophyte phylogeny.

It seems, then, that for the characteristic habit of the earliest land plants (i.e., a Rhyniophyte-like habit) to become established, a mechanism to insure strict apical growth / subapical growth suppression was critical. In our judgement, apical meristems evolved as a consequence of the evolution of a correlative control system that functions to suppress the innate capacity that all intact cells originally had and still have - the capacity to undergo proliferative cell divisions. There is some evidence (Basile 1968) that part of the control system to be described later in this paper plays a critical role in the transition from diffuse to apical growth. Presumably, this same control system underlies the morphogenetic phenomena variously termed "apical dominance," "lateral dominance," and "correlative inhibition."

In the sixty to seventy million years that elapsed between the appearance in the fossil record of *Cooksonia* in the Silurian and the end of the Devonian, the general evolutionary trend was for plants to greatly increase in size and complexity of form and structure. For example, the Rhyniophytes measured millimeters in diameter and centimeters in length. In contrast, the presumed progenitors of extant phyla that evolved during the Devonian measured decameters in diameter and meters in length. This primary trend toward increasing size and complexity continued reaching its fullest expression in the origin and diversification of flowering plants.

In evolutionary theory, it is axiomatic that any new entity must evolve via the modification of some pre-existing entity. Since Rhyniophyte-type plants are the only forms

Fig. 1. Illustrations comparing growth forms of fossil and extant phyla of land plants (Embryophyta). A–D. Rhyniophyta. A. *Cooksonia caledonica* from the Upper Silurian (425 mybp). *Cooksonia* is the most ancient genus of land plants presently known. B. *Steganotheca striata* from the Upper Silurian. C. *Renalia huberi* from the Lower Devonian (395 mybp). D. *Rhynia major* from the Lower Devonian. Note that the axial development of the Rhyniophyte-type plants that appeared during the first 20 million years of land plant evolution (A&B) was symmetrical. Those appearing later, in the lower Devonian (C&D), show a tendency for the sporangium-bearing axes to be subordinate (suppressed?) in their relative development. E. Zosterophyta, *Zosterophyllum* sp. from the Lower Devonian. Some species of *Zosterophyllum* (e.g. *Z. rehenanum*) exhibit strict dichotomous branching of the vegetative axes with only the sporangia formed in a lateral, subordinate position (arrows). F. *Psilotum triquetrum* exhibiting dichotomous branching of the axes and sporangia on short stalks (arrows). G. *Psilotum nudum*, clone sei ryu kaku which does not produce lateral appendages, either sterile or fertile. Sporangia develop instead at the tips of the ultimate branching axes. It appears that the genus *Psilotum* still maintains the morphogenetic capacity to manifest Rhyniophyte-type growth forms. A–D redrawn from Stewart, 1983. E redrawn from Banks, 1970. F redrawn from Bower, 1908. G drawn from Fig. 1 of Rouffa, 1971.

Fig. 2. Illustrations depicting early stages of ontogeny of a chlorophyte and representatives of all eight phyla of extant embryophytes. Note the degree to which development proceeds before apical meristems are established and that the earliest stage of ontogeny among many of the represented taxa are virtually indistinguishable. A. Chlorophyta, *Fritschiella tuberosa*. Vegetative development in this, and most other green algae occurs without the differentiation of apical meristems. B–C. Bryophyta. B. Gametophyte of *Scapania nemorosa*. C. Sporophyte of *Conocephalum* sp. The sporophyte of most, if not all, liverworts go from zygote to reproductive maturity (not shown) without passing through an apical development stage. D. Lycopodiophyta, *Lycopodium* sp. E. Equisetophyta, *Equisetum debile*. F. Psilophyta, *Tmesipteris tannensis*. G. Polypodiophyta, *Gymnogramme sulphurea*. H. Cycadophyta, a–c *Zamia* sp.; d *Macrozamia* sp.; e *Dioon edule*. I. Pinophyta, *Torreya californica*. J–K. Magnoliophyta. J. *Luzula forsteri*, a monocot. K. *Capsella bursa-pastoris*, a dicot. A, C–K redrawn from Wardlaw, 1955. B from Basile, 1990. Drawings are not to scale.

of embryophytes known to have been present for millions of years before others with divergent growth habits appeared, the latter presumably evolved from the former. For example, the earliest deviation from a Rhyniophyte-type habit is the Zosterophyte-type (Banks 1970). The principal difference here is that Rhyniophyta were completely devoid of lateral organs, including sporangia, while Zosterophytes produced sporangia laterally on short stalks. Among extant plants, *Psilotum* characteristically produces sporangia on short branches or stalks, laterally. In this sense, this genus exhibits a Zosterophyte-like habit. But several cultivars of *Psilotum*, including at least one that was obtained from natural populations in Japan, exhibit a Rhyniophyte-like habit in that they are devoid of lateral appendages and form terminal sporangia. (Rouffa 1971, 1973). Rouffa (1978) speculated that terminal sporangia may have been the primitive condition in the Psilotaceae and that their present lateral position may have arisen via genetically based modifications resulting in the "suppressed" development of the sporangium-bearing branch. That is, in a newly initiated bifurcation of a plant axis where one fork will develop into a sporangium and the other remain vegetative, the vegetative fork becomes dominant and appears to suppress axial growth in the other fork. If this is so, it is analogous to what occurs with respect to cotyledon development in all species of the genus, *Streptocarpus*, and the subfamily, Cyrtandroidae, to which it belongs. In these plants both cotyledons are equally developed within the seed. After germination, one of the cotyledons becomes much enlarged (accrescent) while the other remains small or dies. It has been shown experimentally (Rosenblum and Basile 1984) that the development of the cotyledon which remains small is due to its being "suppressed" by the dominant, accrescent cotyledon. It, therefore, does not seem too far fetched to propose that the morphogenetic basis for the forms and symmetries manifest in fossil plants that followed after the origin of the Rhyniophyte-type could have resulted from genetically based modulations in the chemoregulation of either the growth suppressing or the growth maintaining/promoting systems, or both. That is, plant morphologies after the Rhyniophyte-type derive from modifications of the system responsible for initiating and maintaining strict apical, polar, axial growth. Given the hypothesis that the Rhyniophyte-types consist solely of dichotomously branching, terete axes, devoid of lateral appendages, two things may be assumed: 1). Rates and directions of the cell divisions occurring in the populations of cells comprising Rhyniophyte-type apical meristems were uniform and symmetrical throughout. 2). Control of subapical suppression of cell divisions was also uniform and symmetrical throughout. Under these assumptions any mutation which directly or indirectly perturbed the synchrony and symmetry of apical divisions, or the synchrony and symmetry by which chemoregulation of sub-apical suppression was achieved, could result in morphogenetic pattern and form changes. Stebbins (1974, 1986) has repeatedly pointed out that mutation based changes in "meristematic capital" (meristem size) or "mitotic frequency" (meristematic activity) can profoundly alter morphogenetic pattern and form in plants. Are Zosterophyte-type vs Rhyniophyte-type patterns exhibited in *Psilotum* (Rouffa 1978) correlated with differences in "meristematic capital" or "mitotic frequencies" and/or spaciotemporal shifts in place-dependent suppression in their respective apical meristems? Are factors that mediate synchrony and symmetry of "expressed" growth processes in the meristematic population distinct or related to those that mediate the synchrony and symmetry of "suppressed" growth processes in adjacent cell populations? We are hopeful that our continuing research on the role and control of place-dependent growth suppression will contribute toward providing answers.

Unfortunately, we cannot directly determine the morphogenetic basis for paleontological events. Nevertheless, from the pale-

obotanical evidence, it is clear that after several million years, plants with more complex forms came to coexist with, then eventually replace, the Rhyniophyte-type. According to Banks (1970), an "explosive radiation" of morphological variation and innovation took place in the Devonian. With the exception of flowering plants, the beginning of all major plant groups and just about every type of growth-form present today could be found among the Devonian flora. Therefore, the morphogenetic mechanisms responsible for regulating such diverse patterns and forms—hormonal and otherwise— must have become established by the end of the Devonian, some 345 million years before the present. Like the Genetic Code, once a workable, dependable system evolved, it persisted, embellished perhaps, but, essentially unchanged to the present. It is our conviction that the evolution and diversification of form in land plants, then as now, must have the same morphogenetic bases.

Counter to the primary trend toward increased size and complexity cited above is a recurrent secondary tendency to reverse the trend. Among comparative morphologists this secondary tendency is sometimes referred to as "reductive" or "regressive" evolution. It has been reasonably assumed, by some, that in those plants in which size and/or complexity was reduced when compared to the ancestral state, the capacity to achieve the ancestral state was evolutionarily lost and, therefore, developmentally lacking. One important aspect of reductive evolution in phylogeny is pointed out by Stebbins (1992) in discussing evolutionary trends in the reproductive structures of flowering plants. He lists four kinds of morphological changes that are repeated in many evolutionary lines and for which understanding of the underlying control mechanisms is basic to understanding angiosperm evolution. The first two of the four involve evolutionary reduction through loss: (1) the loss (elimination) of all of one kind of part; and (2) the loss (reduction) in number of similar parts. It is probably safe to assume that the mechanism(s) underlying the loss of parts is the same for flower parts as for vegetative parts. In any event, it is in relation to the phenomenon of "loss of parts" that we propose that place-dependent suppression of cell proliferation has played a second, critical role in plant morphogenesis and phylogeny. This proposition is based on experiments conducted with developing gametophytes of leafy liverworts and developing sporophytes of flowering plants that presumably have reached their present morphological state through reductive/regressive evolution.

Streptocarpus is a genus of flowering plants that provides an excellent example of evolution via the reduction and "loss" of parts. At the presumably primitive end of the series are plants such as *S. nobilis* with a well defined root-shoot polarity and the shoot composed of nodes and internodes. At the most derived end are plants like *S. grandifolia* and *S. wannii* with an ill-defined root-shoot boundary and the shoot portion represented by a single leaf-like structure called a phyllomorph. Species in which the vegetative body consists of a single phyllomorph are termed unifoliates. Those in which it consists of a series of phyllomorphs are called pleurifoliates. In both unifoliates and pleurifoliates, the absence of nodal organization is correlated with the failure of plumular meristems to differentiate in either the embryonic or germinated seedlings, coupled with the continued growth, development and differentiation of one of the two cotyledons which forms the phyllomorph (Fig. 3). From comparative studies, it might reasonably be assumed that in the course of speciation, unifoliate and pleurifoliate species of *Streptocarpus* lost and, therefore, lack the capacity to differentiate meristems that generate a node-internode-type organization. Experiments conducted in our lab, however, indicate otherwise (Rosenblum and Basile, 1984; Basile and Basile, unpublished). It was found that if seedlings of a pleurifoliate, *S. proxilis*, were treated with either gibberellic acid, or any of three auxin antagonists, or

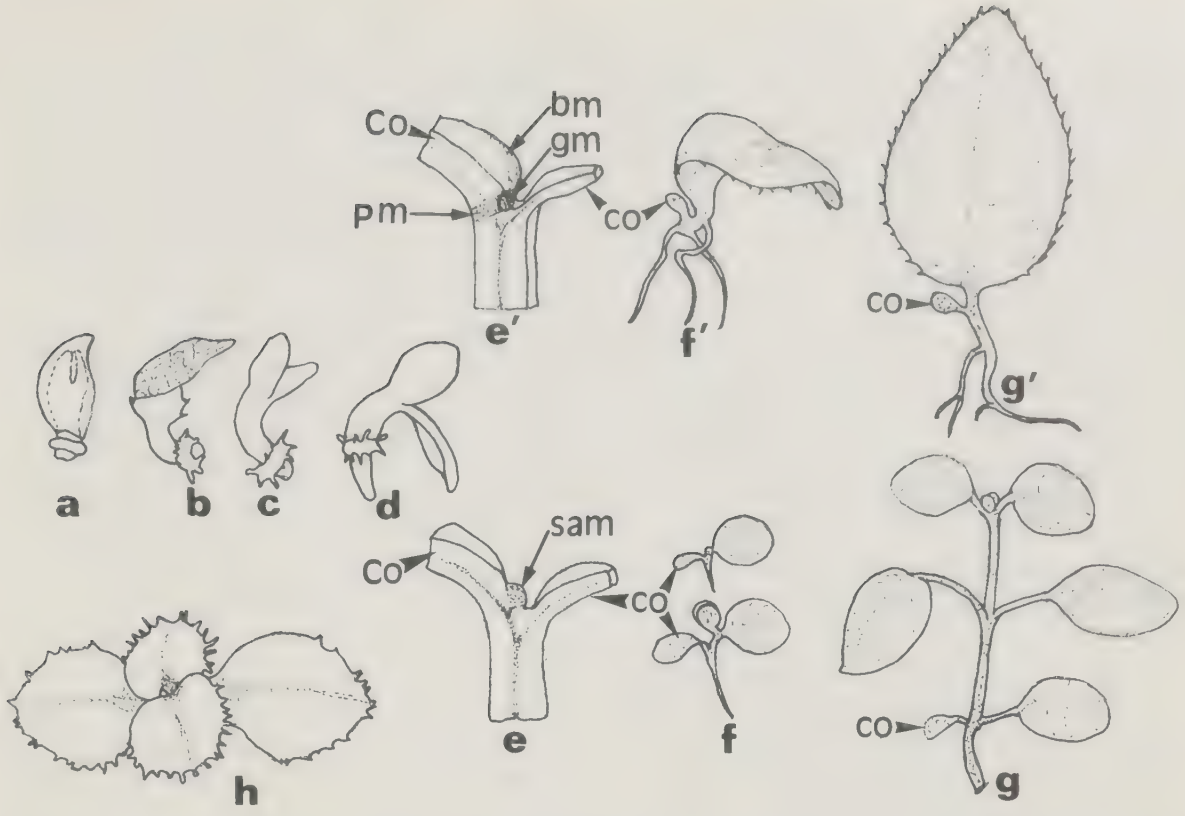

Fig. 3. Drawings to show pertinent stages in the ontogeny of normal and phenovariant *Streptocarpus* seedlings. a–d are stages in seedling development that are essentially the same for all species. e–f are stages in the development of species assigned to subgenus *Streptocarpella*, all members of which eventually manifest nodal organization (g) after passing through an anisocotylous stage (f) in which growth of one cotyledon (Co) is dominant over the other (co). A displacement of the shoot apical meristem (sam, f) is correlated with the accrescence of the larger, "macrocotyledon" (Co). e'-f' are stages in the development of species assigned to subgenus *Streptocarpus*, no member of which manifests true nodal organization. Rather, all members of this subgenus manifest a "phyllomorph"-type organization (f', g') after passing through a stage in which the embryonic shoot meristem is suppressed (e'), its position occupied by inactive meristematic-appearing cells, the groove meristem (gm). An intercalary meristem, the "petiolode meristem" (pm) extends across the hypocotyl from the groove meristem (f'). Cell maturation both proximal and distal to this meristem gives rise to the axial, "petiolode" portion of the phyllomorph. Another zone of intercalated meristematic cells, the basal meristem (bm), develops across the basal, proximal portion of the macrocotyledon (f'). All tissues of the lamina portion of the phyllomorph, exclusive of the midrib are derived from the basal meristem. That anisocotyly and phyllomorph organization follows from the suppression of the embryonic shoot meristem is shown by desuppressing the embryonic shoot meristem of *S. prolixus* by means of auxin and ethylene antagonists. Antagonists of either auxin transport or action, and antagonists of ethylene synthesis or action all have the same effect. The seedlings exhibit isocotyly and desuppressed activity of the shoot apical meristem as manifest in the formation of paired stem leaves (h).

any of three ethylene antagonists, before either of the two cotyledons dominated development, the cotyledons developed equally. These plants also differentiated shoots with nodal organization (Fig. 3). Further details of these experiments are deferred to the portion of the paper concerned with the control of place-dependent suppression. The important point here is the unequivocal evidence that the capacity to develop shoots with node-internode-type organization is neither evolutionarily lost nor developmentally lacking in at least one and probably all species that normally develops a phyllomorph-type vegetative body. It is merely suppressed.

The discovery that the "loss" of nodal organization does not necessitate a mutational nullification or loss of specific alleles required for shoot initiation but rather is due to the time and place of action of certain broad spectrum growth regulators has

two important implications for understanding the controls of plant morphogenesis. For one, it supports Stebbins' (1992) assertion that genotype-phenotype interactions are indirect and epigenetic. That is, "based on successive interactions between regulators produced at various developmental stages, each being integrated with those that precede and that follow." For the other, it supports our "suppression hypothesis" (Basile and Basile 1984) which asserts, "that many if not most of the differences by which we distinguish the form changes that occur during the stages of ontogeny and the course of phylogeny are due to differences with respect to the time and place that cell division and/or cell enlargement is suppressed." The experiments showed both that place-dependent suppression of nodal growth has a major role in speciation of *Streptocarpus* and that place-dependent suppression of microcotyledonary growth plays a distinctive role in the ontogeny of all species assigned to the Cyrtandroidae as well as in the phylogenetic separation of this subfamily from the rest of the Gesneriaceae.

The studies conducted with *Streptocarpus* did not produce the only or first experimental evidence to support this hypothesis. Earlier, more extensive work had been carried out with developing gametophytes of leafy liverworts. The leafy liverworts (Jungermanniales), exemplify a whole order of plants that exhibit a general trend toward reductive evolution (Fig. 4). As a group, leafy liverworts are not only capable of producing leaves in series, but also have the potential for producing 13 distinct types of branching - dichotomous (Basile & Basile 1980b) and 12 types of monopodial branching (Crandall, 1972; Thiers, 1982). In leafy liverwort gametophytes, as with angiosperm flowers, there are two recurring evolutionary trends within the families and genera that involve an apparent loss; one of "number" and the other in "kind" of parts. Those taxa placed at the primitive end of the phylogenetic /morphological series are those with shoots exhibiting radial symmetry with leaves arranged in a 1/3 phyllotaxy and producing frequent and varied types of branching. At the other end, the shoots are bilaterally symmetrical with a 1/2 phyllotaxy and branching lacking, or limited to one type. Thus the general tendency is toward both a loss in the number of leaves and in the kinds of branches.

The change in symmetry and phyllotaxy is most directly related to the fact that one of each series of three leaf primordia formed at the shoot apex fails to develop (Fig. 5). Again, it might reasonably be assumed that the derived condition resulted from the lost or lacking capacity of cells of certain leaf primordia to divide further. Again, experiments conducted in our lab indicate otherwise (Basile, 1990; Basile and Basile, 1990; and literature cited therein). It was found with representatives of five families of leafy liverworts, that if gametophytes that normally have one of each series of three leaf primordia suppressed are experimentally treated at the juvenile stage of development, that is, at a stage transitional between diffuse growth and apical growth, the capacity of all leaf primordia to fully develop was expressed (Fig. 6–7). As with *Streptocarpus*, the place-dependent suppression of cell proliferation in specific leaf primordia has resulted in phylogenetically significant alterations of morphogenetic patterns in leafy liverworts.

The results of these experiments leave little doubt that in the genus, *Streptocarpus*, and the order, Jungermanniales, place-dependent suppression of cell proliferation plays an important role in the phylogenetic trends toward "reductive" evolution through the "loss" of parts. One striking difference between desuppressing shoot primordia in *Streptocarpus* and desuppressing leaf primordia in leafy liverworts were the concommitant changes in branching patterns and leaf morphology that often accompanied desuppressing leaf development in the liverworts. In all species we work with, but especially *Scapania nemorosa*, both the number (frequency) and kinds of branches that developed was greatly increased when

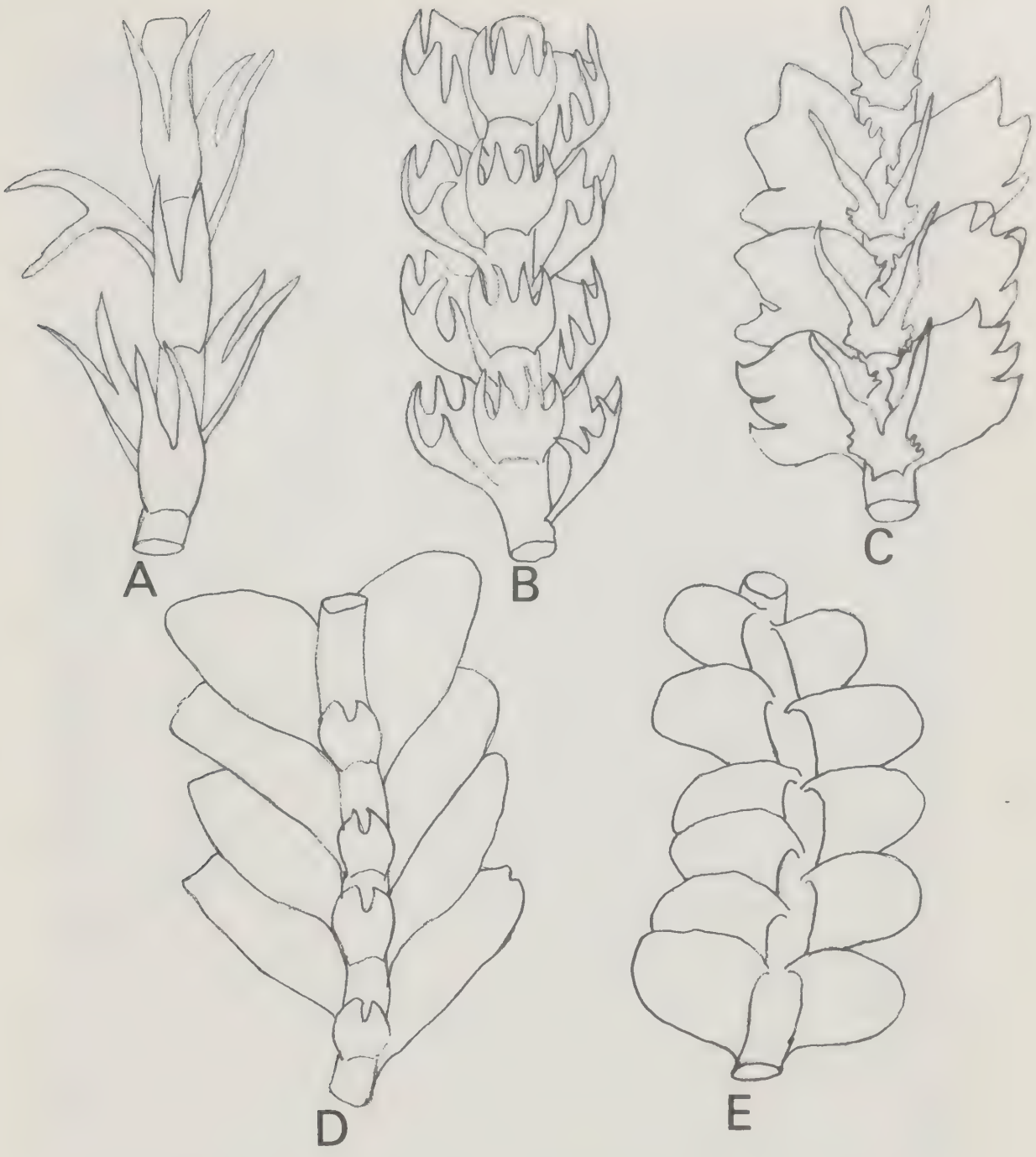

Fig. 4. "Reductive evolution" in the Jungermanniales. A–E illustrate a reduction series with respect to leaf development among the different families of leafy liverworts (see also Fig. 5). A. *Herberta* sp. (Herbertaceae) with each leaf of a three leaf sequence developing equally. B. *Lepidozia* sp. (Lepidoziaceae) with one of every three leaves somewhat smaller. C. *Lophozia* sp. (Lophoziaceae) and D. *Calypogeia* sp. (Calypogeiaceae) with one of every three leaves appreciably smaller. E. *Jungermannia* sp. (Jungermanniaceae) with every third leaf "lacking." Similar reduction series may be exhibited within single families such as the Lophoziaceae.

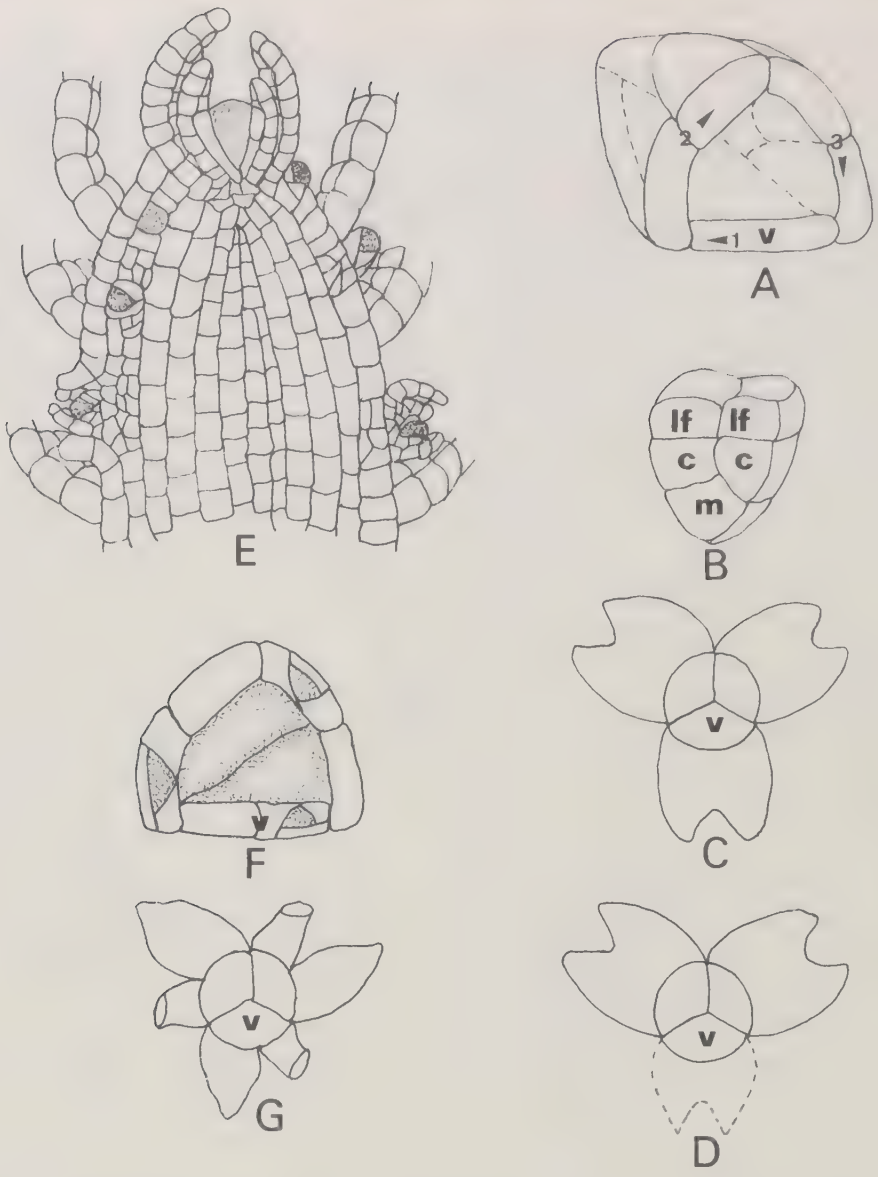

Fig. 5. Diagrams to show the potential sites of initiation of leaf and branch primordia that become "lost" through suppression during the course of "reductive evolution". A-D. Leaf initiation. A. Apical cell with three segments (merophytes) "cut off" in clockwise sequence. In plants with creeping or suberect growth habits, the merophytes toward the substratum are termed ventral (v), the others lateral. Each merophyte divides to form two (A2), then three (A3) cells. The two anterior cells divide again to form a five celled merophyte. B. The developmental fate of the five cells resulting from the divisions just described are as follows: medullary (m) and cortical (c) initials of stem tissues and paired leaf (lf) initials. C. Radial symmetry that results if all three merophytes contribute equally to stem and leaf development. D. Bilateral symmetry that results if further cell division of the ventral leaf primordia is suppressed. E-G. Branch initiation. E. Relative positions that lateral branches may be initiated via special partitioning divisions of stem cells. Stippled cells at left indicate the position where endogenous branches may arise through the transformation of internal cortical cells near the stem apex. Stippled cells at right similarly indicate where exogenous branches may arise. Depending on whether the branches arise from ventral or lateral merophytes and, if the latter, whether they mature in a position adaxial or abaxial or closer to the anterior or posterior margins of the lateral leaves they are given different names. F. Relative positions that terminal branches may be initiated either by a sequence of three special partitioning divisions of one of the paired leaf primordia produced by each merophyte, or by a bipartitioning of the apical cell itself. Bipartitioning of the apical cell leads to true dichotomous branching, the most primitive type for land plants. It is apparently very rare in nature and has only been observed by us in plants that were experimentally desuppressed. G. Three types of terminal branches distinguished by whether they are initiated from a ventral (v) leaf primordium, the ventral "half" or the dorsal "half" of a lateral leaf primordium. It must be emphasized that although certain branch types are characteristically produced by various taxa of leafy liverworts, branch primordia are not produced in the same regular, predictable sequence as are leaf primordia.

Fig. 6 Desuppression of ventral leaf development in representatives of three families of leafy liverworts that manifest "reductive evolution" via the "loss" of parts. A&B. Suppressed and desuppressed plants, respectively, of *Scapania nemorosa* (Scapaniaceae), ventral view. C&D. Suppressed and desuppressed plants, respectively, of *Nowellia curvifolia* (Cephaloziaceae), ventral view. E&F. Suppressed and desuppressed plants, respectively, of *Jungermannia leiantha* (Jungermanniaceae), ventral view. Although not shown here, the capacity to produce branch types thought to be "evolutionarily lost" was experimentally desuppressed along with ventral leaf development.

Fig. 7. Desuppression of ventral leaf development in representatives of two families of leafy liverworts that manifest "reductive evolution" via the "loss" of parts. A&B. Suppressed and desuppressed plants, respectively, of *Plagiochila arctica* (Plagiochilaceae), ventral view. C&D. Suppressed and desuppressed plants, respectively, of *Gymnocolea inflata* (Lophoziaceae), ventral view. Although not shown here, the capacity to produce branch types thought to be "evolutionarily lost" was experimentally desuppressed along with ventral leaf development.

leaf primordia were experimentally desuppressed (Fig. 8). The mechanism by which branch development is desuppressed cannot be the same as for leaves. Branch primordia are not layed down in a regular sequence as are leaf primordia. In fact, branch primordia are not laid down at all. They are initiated by a special sequence of partitioning divisions of either leaf primordial cells, or epidermal or cortical stem cells, or of the apical cell itself. One explanation for this "indirect desuppression" of branch initiation that comes to mind is based on the biophysical principles and models put forward by Lintilhac (1974) and Green (1986, 1987). Both authors assert that the direction or position of cell plate formation within dividing cells is largely determined by the tensile and compressive forces imposing stresses on the cell.

Fig. 8. Branching types produced by experimentally "desuppressed" plants of *Scapania nemorosa* (L.) Dum. A. *Anomoclada*-type (lateral, endogenous). B. *Bazzania*-type (lateral, endogenous). C. *Acromastigium*-type except the branch appears to replace the whole rather than half a ventral leaf (terminal). D. *Bryopteris*-type (lateral, endogenous). E. *Plagiochila*-type (lateral, endogenous). F. *Frullania*-type (terminal). G. *Radula*-type (lateral, exogenous). Only the *Plagiochila*-type (E) is characteristic of *S. nemorosa*. (From Basile, D.V. 1974. J. Hattori Bot. Lab. 38: 91-98, with permission).

Lintilhac (1974) proposed the principle that the new cell plate aligns perpendicularly to the greatest stress passing through the cell, in other words, in the plane that is most free of shear forces. To these ideas Green adds that the initiation of organs at organized shoot apices operates pretty much as a machine propelled by the rhythmic pattern of cell division and cell enlargement and the concommitant rhythmic changes in distribution of stretching and compression of cells therein. It follows from the foregoing that by experimentally desuppressing cell proliferation in one zone of the shoot apex, the rhythm of division and enlargement, and, therefore, the pattern of tensile and compressive forces propagated through the growing region were significantly changed. This change in the distribution of forces apparently either stimulated or permitted the changes in cell partitioning that transforms an erstwhile stem cell, leaf initial, or apical cell into a branch primordium (Fig. 5). Until we have some means of measuring the force-fields or changes thereof in developing shoots of the liverworts we work with, we cannot test this hypothesis. Nevertheless, whether by elimination ("loss") of a kind of part (a specific branch-type) or by a reduction ("loss") in number of parts (leaves), our experiments show reductive evolution in the Jungermanniales to be different manifestations of place-dependent suppression of cell proliferation and enlargement. We expect that in time it will be possible to experimentally demonstrate in other groups of plants manifesting reduction series via the "loss" in kinds and/or number of parts that the capacity to produce the parts is not lost or lacking - just suppressed.

Besides its roles in effecting the transition from diffuse to apical growth and in the recurrent trend toward "reduction" in numbers and/or kinds of parts, place-dependent suppression of growth has a third important role in plant morphogenesis and phylogeny. This is the modulation of size and shape of organs of determinate growth. Support for this can easily be obtained by comparative developmental studies of closely related plants or even ontogenetic studies of a single species. Because most of our research on plant morphogenesis has been conducted with leafy liverworts, they will serve as our examples.

Scapania nemorosa is one of a great many liverworts that manifests heterophylly during its ontogeny (Fig. 9). The first leaves formed are plane and bifid, as is the case for most leafy liverworts. As successive leaves are formed it can be seen that the later formed leaves are larger than the earlier—not because the cells are larger but because there are more of them. The size difference then is due to the suppression of cell proliferation sooner in the smaller first-formed leaves than in the larger ones initiated later. The next morphological change noticeable in the sequence of leaves produced is in their shape. One of the two lobes of the bifid leaves becomes progressively larger with each successive leaf. As a result, successive leaves not only differ in size but also in shape. Bopp and Feger (1961) analyzed cell division patterns in a large number of leafy liverworts and found that the great variety of leaf forms characteristic of the Jungermanniales is reducible to five variations with respect to where and when cell divisions stopped (were suppressed) in subpopulations of the total population of cells comprising the developing leaves. Apparently, the same regulatory system that modulates the suppression/desuppression of leaf and branch primordia in leafy liverworts, modulates the suppression/desuppression of cell division in developing leaves. In the same experiments in which leaf primordia and branch initiation were desuppressed, the spacial and temporal patterns of growth suppression that occur during the development of individual leaves were also altered. The result was that leaf sizes and shapes were often profoundly changed; so much so that the experimentally modified plants have been described as "Phyletic Phenocopies" (Stebbins and Basile 1986) since they closely resembled the normal form of plants

Fig. 9. Heterophylly in *Scapania*. Several sizes and shapes of leaves are produced (e.g., plane, bifid, (a); concave, bifid, (b); conduplicate, (c) during the course of ontogeny before the complicate-bilobed leaves (d) typical for the genus are formed.

belonging to different genera and families of leafy liverworts (Fig. 10). Certainly, modulation in the time and/or place that cell proliferation is suppressed in a developing, determinate organ such as leaves is not the sole basis for size and shape change. Changes in rate and direction of cell division and rate and direction of cell enlargement are extremely important, being two of the "driving forces" in plant morphogenesis. Spaciotemporal changes in place-dependent suppression does, nonetheless, play an important role in modulating size and shape changes of organs and thereby has a third and generally important role in morphogenesis and phyllogenesis.

To better understand and investigate the role of place-dependent suppression of cell proliferation in morphogenesis and speciation, it will be necessary to better understand the means by which it is controlled. The above cited experiments that showed that cell proliferation that is normally suppressed in specific cell populations can be desuppressed also have provided useful information about the chemical regulation of place-dependent suppression of cell division. That information is summarized as follows:

AUXIN →

(directional transport of auxin determines *where* suppression occurs)

ETHYLENE →

(auxin-triggered ethylene synthesis determines *when* suppression occurs)

HYDROXYPROLINE-CONTAINING GLYCOPROTEIN

(ethylene-mediated modification with respect to hydroxyproline-containing protein (hyp-proteins) associated with the cell surface determines *whether* and *how* suppression occurs)

The experiments indicated that one way by which place-dependent suppression may be regulated is by the interrelated actions of auxin, ethylene, and an hydroxyproline-containing protein. This scheme is based on a long series of experiments in which antagonists supplied at critical stages in ontogeny resulted in the desuppression of cell division in specific populations of cells. In these experiments, at least three different antagonists of each implicated regulator was found to produce the same effect: cell proliferation was desuppressed in populations of cells in which it was normally suppressed. Because each antagonist for any one of the growth regulators was structurally different and/or had a different mode of antagonism, we are very confident that auxin, ethylene and an Hyp-protein are components of the "suppression system." Besides identifying important components of the regulators of growth suppression, the experiments indicate that the "critical" stage both in *Streptocarpus* and in the liverworts is one that is transitional between the

Fig. 10. Variations in leaf morphology resulting from the experimental "desuppression" of developing gametophytes of *Scapania nemorosa* (L.) Dum. Some of the forms of leaves produced from lateral merophytes are illustrated in A-C,F,G,I,K,L. Only A is characteristic of *S. nemorosa*. Some of the leaves formed from ventral merophytes are illustrated in B,D,E,H,J. Ventral leaves are not characteristic of the genus (*Scapania*) of the family (Scapaniaceae) to which it belongs. In fact, most of the leaves illustrated are not typical of the genus or family, but phenocopy those of other species belonging to other possibly more primitive/less derived families of the Jungermanniales. Note that there is a greater tendency for both lobes of the "desuppressed" leaves to develop more symmetrically. (See also leaves illustrated in Fig. 8). Redrawn from Basile, D.V. 1974. J. Hattori Bot. Lab. 38: 91-98, with permission).

pre-apical (diffuse growth) stage and apical meristem forming stage in ontogeny. We think this finding is highly significant. Apparently this is a phase in which the regulatory system for maintaining dominance (apical in liverworts and lateral in *Streptocarpus*) is just becoming established and is not fully effective.

In *Streptocarpus*, antagonists of either auxin transport or action and of either ethylene synthesis or action resulted in desuppressed microcotyledonary development resulting in isocotyly and in plumule initiation leading to a nodal organization of the vegetative plant body. Experiments to see if antagonists of hydroxyproline-protein synthesis will have the same morphogenetic effects are in progress. In the five representative leafy liverworts, the same auxin and ethylene antagonists used with *Streptocarpus*, plus five antagonists of hydroxyproline-protein (Hyp-protein) synthesis resulted in desuppressed leaf and branch development as well as altered leaf morphologies. Antagonism of Hyp-protein synthesis also was found to prevent the transition from the diffuse to the apical growth stage in a leafy liverwort and two mosses (Basile 1968 and literature cited therein). Although the study with *Streptocarpus* is still incomplete, the available results are sufficient to indicate that the same chemoregulatory system functions to locally suppress cell proliferation in leaf primordia of liverworts and of shoot apical meristems in flowering plants. And at least one of the three components, Hyp-protein, mediates the suppression that leads to establishment of apical growth in bryophytes. The morphogenetic consequences of altering the interrelated action of this one system is time- and place-dependent.

The evidence that auxin, a well-established promoter of both cell enlargement and (with cytokinin) cell proliferation, is part of an integrated regulatory system is significant, even if predictable. Because its transport through organized plant tissues is strongly directional and its effective concentrations subject to a high degree of regulation, auxin is the component of the system most likely responsible for spacial ordination. That is, where it is transported in a sufficiently effective concentration to trigger ethylene production is critical to establishing the spacial component of the spaciotemporal regulation of growth suppression. What accounts for the time during development that different cell populations become differentially responsive to any given auxin concentration is still an enigma. It is probably safe to assume that the differential sensitivity will reside in subtle differences in the physico-chemical make-up of plant cell surfaces.

Of the three components identified as being interrelated in the regulation of growth suppression, the Hyp-proteins are the most likely to contribute toward subtle differences in the make-up of plant cell surfaces. Particularly interesting in this regard are the arabinogalactan proteins (AGPs). This is a large family of hydroxyproline-containing glycoproteins and proteoglycans reported to be regular components of representatives of all extant land plants (Clarke *et al* 1978). More germane to this contribution were the related papers (Clarke and Knox 1978; Clarke *et al* 1979; Fincher *et al* 1983; Van Holst and Clarke 1986) in which it was suggested, on both experimental and inferential evidence, that AGPs may function in cell recognition, cell-cell communication, or morphoregulation. Consequently, we began (Basile *et al* 1986) to test the hypothesis that some "species" in the large family of AGPs plays a key role in morphogenesis, specifically in the place-dependent suppression of growth. We tested it first, indirectly, by trying to ascertain that AGPs were not only present in the plants, but in all the intact, living cells. That is, cells in which the potential for further growth via cell proliferation and/or enlargement was alledgedly "suppressed." We did this by using Yariv's *B*-glucosyl reagent to test representatives of both subclasses and all six orders of liverworts (Basile & Basile 1987; and unpublished), all families of mosses (Mankiewicz and Basile, unpublished), and

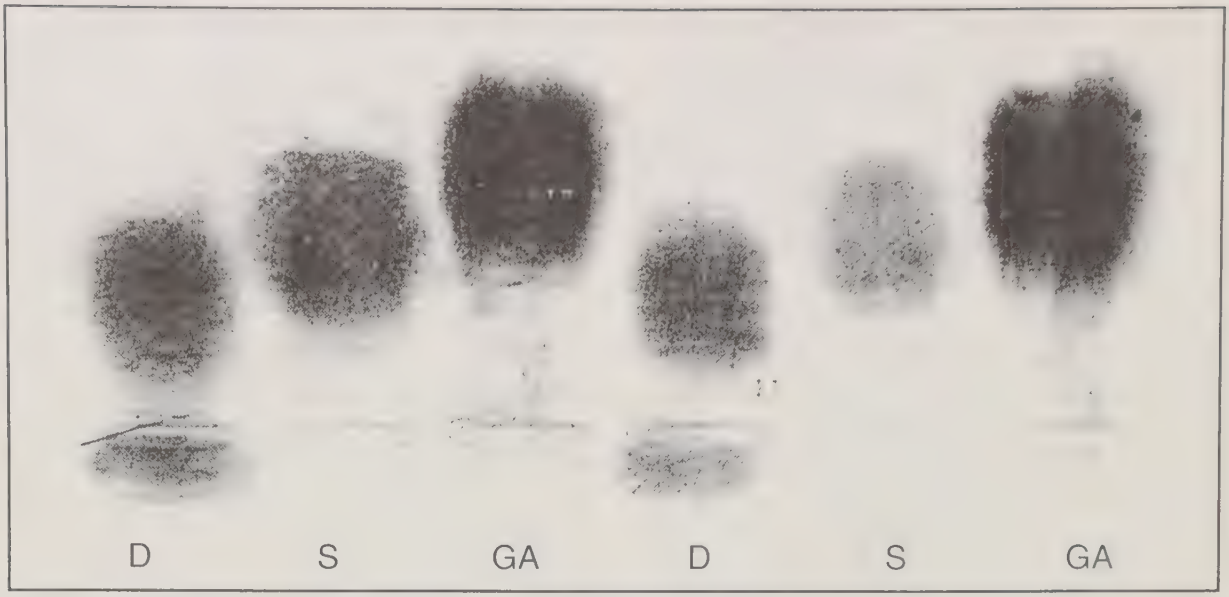

Fig. 11. AGPs from plants with suppressed (S) and desuppressed (D) leaf development compared by the glyoxol gel electrophoresis method of Basile *et al.* (1989) stained with Yariv's *B*-glucosyl reagent and gum arabic (GA) as a standard.

all genera of cycads (Basile *et al* 1990). Yariv's reagent is an artificial antigen that specifically binds, precipitates and stains AGPs red-orange. In all the representatives tested, AGPs were found at the surfaces of all the living cells. It seems highly probable that AGPs are regular components of all plant cell surfaces where considerable regulation takes place. Better evidence for some sort of regulatory role for AGPs than their ubiquitous occurrence at plant cell surfaces is that obtained by Roberts and co-workers (Knox *et al* 1989, 1991; Stacey *et al* 1990; Pennell *et al* 1991). They have produced a panel of monoclonal antibodies to AGP epitopes that proved to be developmentally regulated. The epitopes recognized by the antibodies are expressed in different positions at different times during the course of embryogenesis and organogenesis. The temporal and spatial changes in the expression of these epitopes may precede and, therefore, predict stage-change in embryogenesis and pattern-change in histogenesis. At this point, there is no direct evidence for any AGP epitopes being responsible for cell-inductive processes, but the temporal-spatial sequences of their expression indicate a continually changing environment for relatively fixed populations of cells. It has been suggested that the local environment may be more important than the cell *per se* in the development of tissue pattern formation (Edelman 1986b). While some of the panel of monoclonal antibodies may predict tissue pattern formation, none thus far predicts place-dependent suppression.

In preparation for attempting to develop monoclonal antibodies that specify AGP epitopes correlated with growth suppression, we have been trying to find the appropriate AGPs to use as antigens. We have been fortunate in our discovery of one species of leafy liverwort, *Gymnocolea inflata*, which maintains its normal "suppressed" pattern of leaf and branch development when cultured on ammonium-free nutrient medium and a "desuppressed" pattern on ammonium ion-containing medium (Basile and Basile 1980a) (Fig. 7). The advantage of this is that we can accumulate relatively large amounts of "suppressed" and "desuppressed" plants and the media in which they were growing for comparative analysis of their AGPs. When the AGPs extracted from plants cultured on the two different types of media were compared by agarose gel electrophoresis, a clear difference in the electrophoretic patterns was produced by the two samples (Fig. 11). These results

provided some support to the idea that desuppression could be correlated with some component of the AGPs. But since no sharp banding pattern was observed in these gels, it was impossible to tell which, if any, specific portion of the AGPs had been altered. Therefore, a different method, cesium chloride gradient analysis, was used to separate and compare the glycoproteins. With density gradient analysis, it is possible to separate the AGPs into a relatively large number of fractions based on differences in buoyant density.

When the AGPs, either extracted from the plants or separated from the culture media, were compared by this method some very promising results were obtained. Buoyant density fractions present in extracts from the suppressed plants were absent in those from the desuppressed plants! Conversely, when the AGPs from the culture media were compared, the opposite or reciprocal results were obtained (Fig. 12). The buoyant density class that was absent in the desuppressed plants was now present in the culture medium in which they were growing. The simplest explanation for these results is that, correlated with the process of desuppressing cell proliferation, certain high buoyant density AGPs are released from the cell surfaces and they diffuse into the culture medium.

To see if the apparent release of HBD-AGPs by plants in which cell proliferation was experimentally induced was not restricted to liverworts but a general phenomenon, complementary experiments were conducted with stem pith of lettuce (Mignone and Basile 1992 and unpublished). The result of these experiments showed that HBD-AGPs present in extracts of non-proliferating ("suppressed") stem pith was absent from the proliferating ("desuppressed") pith (Fig. 13). Since the lettuce pith tissues were cultured on agar-solidified media, a comparative analysis of AGPs that may have been released into the culture medium was not attempted.

Because the apparent release of high buoyant density AGPs could be correlated with a change from suppressed to desuppressed cell proliferation in such ontogenetically and phylogenetically separated plants as a liverwort gametophyte and a flowering plant sporophyte, we think it may be a generally occurring phenomenon. More specifically, we suspect that epitopes associated with HBD-AGPs are critical components in a molecular control *system* that acts to help keep cells which are capable of dividing in a suppressed state.

The experimental and analytical procedures hitherto used to arrive at an hypothesized role for HBD-AGPs in place-dependent suppression of growth lack the resolving power to rigorously test the hypothesis. Fortunately, Edelman, having perceived that cell surface modulation was a major mechanism in the regulation of animal form and tissue pattern formation, developed immunological methods that facilitated the identification and isolation of putative morphoregulatory molecules associated with cell surfaces (Edelman 1976; 1986a). Because in animals, "form arises as a result of the differential effects of the driving forces provided by the processes of cell division, cell movement and cell death" (Edelman 1986b), while in plants, form arises as a result of the differential effects of the driving forces of cell proliferation, *directional* cell partitioning, *directional* cell enlargement and the place-dependent suppression of all growth processes, the regulation of the driving forces will be correspondingly different. Nevertheless, the genesis of form in both vertebrate animals and land plants are in many respects *analogous*. In both, the underlying genetic controls are mediated by epigenetic mechanisms. In both, temporal and spatial modulation of cell surface molecules appear to constitute a major mechanism in the regulation of form. Since the compositions of the extracellular matrices in vertebrate animals and land plants are quite different, we can expect the regulatory molecules operating within these matrices to be correspondingly different.

Although the cell surface-associated pro-

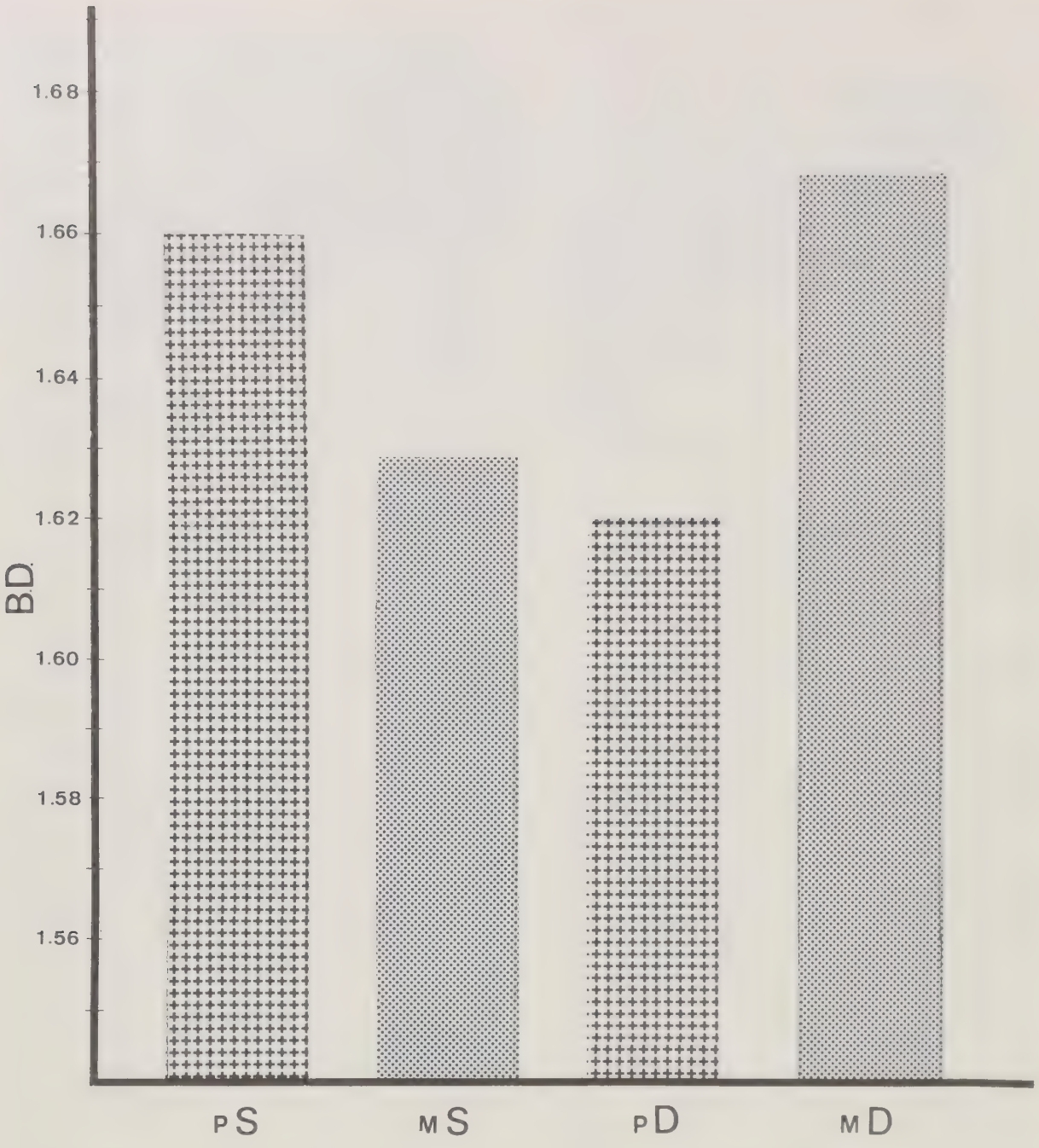

Fig. 12. A comparison of the mean buoyant densities of AGPs extracted from suppressed plants (PS), desuppressed plants (PD), as well as from the media in which they were axenically cultured, (MS) and (MD), respectively. An ANOVA based on six experiments indicated that the differences in mean buoyant density between suppressed plants (PS v PD) and between the media on which they were growing (MS v MD) are statistically significant at 95% probability.

teins involved in modulating morphoregulation in animals and plants may be different, the methods for identifying and isolating them need not be. We have adopted Edelman's approach (1986a) and are presently attempting to produce monoclonal antibodies against the high buoyant density AGPs that are released from *Gymnocolea inflata* plants correlated with the desuppression of cell division in leaf primordia. Our objective is to obtain one or more antibodies which will block suppression. That is, we hope to obtain an antibody that will produce the same effect as the

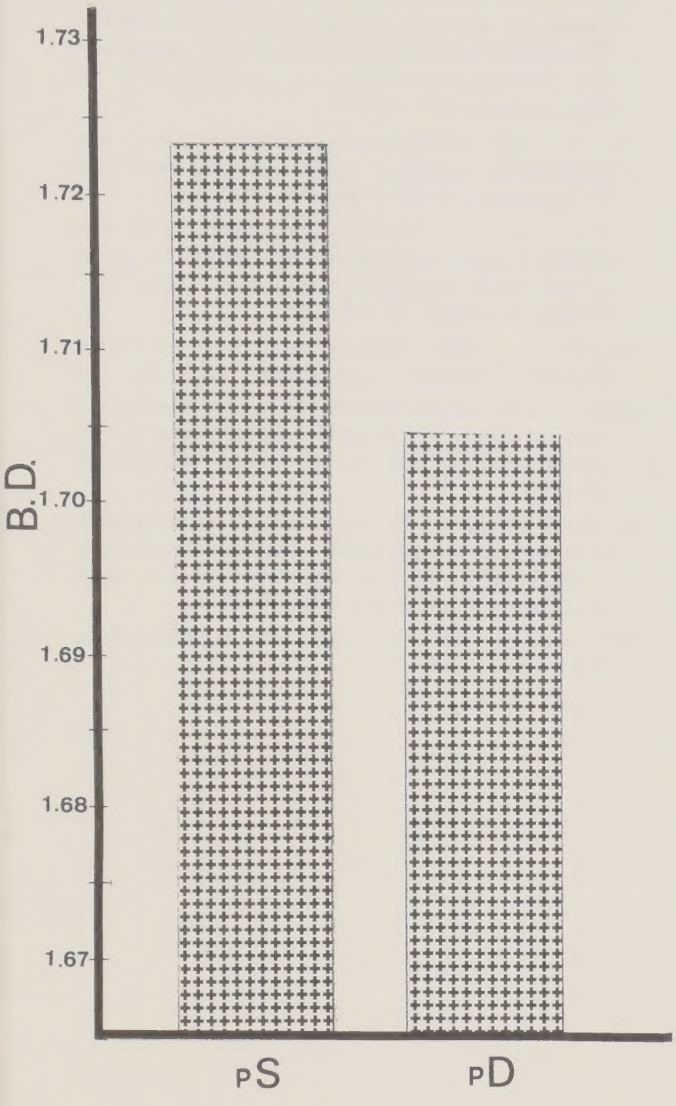

Fig. 13. A comparison of mean buoyant densities of AGPs extracted from suppressed (non-proliferating) (pS) and desuppressed (proliferating) (pD) stem pith of lettuce. An ANOVA based on eight experiments indicated that the differences in buoyant density are statistically significant at 95% probability.

metabolic inhibitors/antagonists used in our earlier experiments. If we succeed, we will have a powerful tool to help identify, isolate and characterize one or more AGPs. We suspect these molecules play a small but critical role in what must be a complex mechanism which differentially selects and maintains potentially dividing cells in an extended state of growth suppression.

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